Are Xenoestrogens Breast Cancer Risk Factors? Unravelling the Cocktail Effect

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by

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Abstract

Breast cancer is an important well-known estrogen receptor (ER) mediated disease. Interactions with ERs rely on salient features of estrogen molecules. If these features occur on other molecules they are likely to mimic estrogens (i.e. xenoestrogens). Until recently, ERs were thought to comprise only a single binding cleft (the ligand binding cleft (LBC)) which determined activity. In 1988, a second binding cleft (known as activation function (AF)-2) was discovered. AF-2 is intimately related to LBC both in its location and function. This thesis explores this relationship in greater detail.

Xenoestrogens were selected for study based on their environmental prevalence. They were subjected to CALUX® assays (a gene reporter assay based on expression of ERs) and MCF-7 (cultured ER+ve breast cancer cells) proliferation studies. Studies were carried out with individual estrogens and xenoestrogens and mixtures representing potential human exposure cocktails. Most combinations showed estrogenic additivity while some xenoestrogens in combinations had anti-estrogenic (ameliorative) effects. This has significant implications for the biological effects of complex cocktails humans are exposed to on a daily basis.

In silico molecular modelling studies (Schrödinger platform) show an intimate connection between LBC and AF-2. This might explain the complex interactions (additive or ameliorative) between xenoestrogens in cocktails at the ER binding clefts. *In silico* studies showed that some xenoestrogens can interact at both the LBC and AF-2 (two-site binding model) and that this has implications for their biological effects. In an ER+ve breast cancer context interactions at ERs could influence cancer cell proliferation depending on the combination of xenoestrogens the person is exposed to.

A descriptive cross-sectional study (n=227) that explored Canterbury (New Zealand) women's and their daughters' exposures to xenoestrogens showed that dietary and environmental xenoestrogens made a significant contribution (mean= 51.3%) to the total estrogenic load in women and was greater (58.1%) in their daughters. In a separate study blood samples were taken from women (n=47), and analysis (high performance liquid chromatography-mass spectrometry (LC-MS)) showed the presence of parabens (100.0% of women studied), phytoestrogens (93.6%) and EE2 (2.1%). These results show that women were both exposed to and displayed blood levels of xenoestrogens. This could have implications in a breast cancer risk context.

This thesis concludes that women and pre-pubertal girls are exposed to complex combinations of xenoestrogens. The combined estrogenic effects signal unscheduled (i.e. occurring before the scheduled time) cellular ER-mediated events; e.g., the earlier onset of puberty, and proliferation of ER+ve breast cancer cells. This is made more complex by the two-site binding model which can lead to amelioration of estrogenic effects. Thus, predicting xenoestrogen cocktail effects in a breast cancer risk setting is at best almost impossible. Further research will increase our understanding of the two-site binding model and will provide opportunities for reducing exposure to breast cancer risk factors and perhaps developing new treatments for breast cancer.

For Aunty Jenny
1946-2010

"When someone you love becomes a memory, the memory becomes a treasure"

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List of Abbreviations

	Abbreviation
1, 1, 1-Trichloro-2, 2-bis(p-chlorophenyl)ethane	DDT
1, 2-diaminocyclohexane-N, N, N', N'-tetraacetic acid	CDTA
17α-Ethinyl estradiol	EE2
17β-Estradiol	E2
2, 2-Bis(4-chlorophenyl)-1, 1-dichloroethylene	DDE
3-Dimensial	3D
4-Hydroxytamoxifen	4-OHT
Activation function-1	AF-1
Activation function-2	AF-2
Activation protein 1	AP-1
Adenosine triphosphate	ATP
American type culture collection	ATCC
Androgen receptor	AR
B-cell lymphoma 2	Bcl2
Bcl-2-associated X protein	BAX
Bisphenol A	BPA
Breast cancer 1	BRCA1
Breast cancer 2	BRCA2
Chemically activated luciferase expression	CALUX®
Christchurch District Health Board	CDHB
Concentration addition	CA
Cyclic adenosine monophosphate	cAMP
Cyclin dependent kinase 2	CDK2
Diethylstilbestrol	DES
Dimethyl sulfoxide	DMSO
Dithiothreitol	DTT
DNA binding domain	DBD
Dulbecco's Modified Eagle Media	DMEM
Dulbecco's Modified Eagle Media: Nutrient Mixture F12	DMEM/F12
E6 associated protein	E6AP
Epidermal growth factor receptor	EGRF
Estrogen equivalent	EQ
Estrogen receptor	ER
Estrogen receptor negative	ER-ve
Estrogen receptor positive	ER+ve
Estrogen receptor α	ERα
Estrogen receptor β	ERβ
Estrogen response element	ERE
Ethylenediaminetetraacetic acid	EDTA
Extracellular regulated kinases	ERK
Fetal bovine serum	FBS
Fructose-1, 6-bisphosphate	FBP1
Fructose-1, 6-bisphosphate 2	FBP2
G protein-coupled estrogen receptor	GPER

Glucocorcoid receptor	GR
Glucose-6-phosphate dehydrogenase	G6PD
Heat shock protein	HSP
High performance liquid chromatography	HPLC
High performance liquid chromatography-mass spectrometry	LC-MS
Hormone replacement therapy	HRT
Human bone osteosarcoma epithelial cell line	U2OS
Human epidermal growth factor receptor 2	HER2
Induced fit docking	IFD
Insulin like growth factor	IGF
Insulin like growth factor 1	IGF-1
Insulin-like growth factor 1 receptor	IGF-1R
International Union of Pure and Applied Chemistry	IUPAC
Intrinsically disordered	ID
Knowledge base for estrogen responsive genes	KBERG
Ligand binding cleft	LBC
Ligand binding domain	LBD
Michigan Cancer Foundation-7	MCF-7
Minimum Essential Media	MEM
Mitogen-activated protein kinase	MAPK
No observable adverse effect level	NOAEL
No observable effect concentration	NOEC
Non-essential amino acid	NEAA
Oral contraceptive	OC
PBS/EDTA	PE
Pentose phosphate pathway	PPP
Phenol red free	PRF
Phosphatase and tensin homolog	PTEN
Phosphate buffered saline	PBS
Phosphoimositide 3-kinase	PI3K
Phospholipase C	PLC
Progesterone receptor	PR
Protein Data Bank	PDB
Protein kinase A	PKA
Protein kinase C	PKC
RAC-alpha serine/threonine-protein kinase	AKT
Rigid receptor docking	RRD
Rose Park Memorial Institute-1640	RPMI-1640
S-phase kinase associated protein 2	SKP2
Selective estrogen receptor modulator	SERM
Specificity protein	Sp
Statistical analyser system	SAS
Steroid hormone binding gobulin	SHBG
Steroid receptor coactivator	SRC
Structure activity relationship	SAR
Sulfotransferase	SULT
The Food and Drug Administration	FDA
Tricarboxylic acid	TCA
Union for International Cancer Control	UICC
University of Canterbury	UC
Uridine 5’ diohospho-glucuronosyltransferase	UGT
World Health Organisation	WHO

Worst case scenario	WCS

Glossary

Estrogens: the female sex hormones; refers to a group of three compounds (estrone, estradiol and estriol). Estrone is found in women and men in small amounts. It is produced in the adrenal gland and produced and stored in fatty tissue. Estradiol (E2) is the most active of the three compounds. It is produced by the ovaries in women and by the testes and adrenal glands in men. Estriol is the major estrogen produced during pregnancy. E3 is produced in large amounts in the placenta and levels in the mother and fetus rise continuously until just before birth.

Androgens: the male sex hormones; refers to a group of five compounds, of which testosterone and dihydrotestosterone are the primary active hormones. Dihydrotestosterone is the metabolite of testosterone and is a more potent androgen than testosterone because it has a higher binding affinity for the androgen receptor. The other three androgens are intermediates in steroid hormone biosynthesis.

Agonist: a compound that is able to bind to a receptor in a cell and produce the biological response that is controlled or triggered by the receptor. The ability to bind is known as ***affinity***. The ability to produce the biological response is known the ***efficacy***. An agonist can be endogenous such as the sex hormones or exogenous substances such as drugs or other chemicals. For the sex hormone receptors, the biological response that is of interest is gene transcription. For the sex hormone receptors, agonists activate (initiate, upregulate) receptor mediated transcription.

Antagonist: a compound that is able to bind to a receptor in a cell and blocks or inhibits the biological response that is controlled or triggered by the receptor. An antagonist has *affinity* for the receptor but no *efficacy*. For the sex hormone receptors, antagonists block (inhibit, downregulate) receptor mediated transcription

Agonist and **antagonist** are also used to describe the 3D conformation of the ER protein ligand binding domain (LBD) on ligand binding, specifically the difference in position of a-helix 12 (H12) of the LBD that occurs when an agonist or antagonist

ligand is bound; Thus, a ligand can be described as an agonist or antagonist and the ER can be described as taking an agonist or antagonist conformation.

Selected estrogen receptor modulators (SERMs): a class of compounds that interact with the ER but unlike pure agonists or antagonists their mode of action depends on the cell type (tissue) they are acting on, resulting in selective inhibition or stimulation of estrogen-like action in the tissue.

Xenoestrogen: a compound that an organism is exposed to via interactions that promote or interfere with ER-mediated gene transcription via binding with the ER. These include natural and synthetic estrogen mimicking compounds and exogenous naturally occurring estrogens.

Coregulatory protein: a cellular protein that interacts with the ligand bound receptor at the ERE that enhances or suppresses gene transcription. Coregulator proteins that enhance or increase the rate of gene transcription are called coactivators and those that supress or decrease the rate of gene transcription are called corepressors. Coregulator protein activity is regulated by their absolute and relative expression levels in different cell types. Coregulator proteins allow for cell type and promoter specificity for transcription factors.

Genomic signalling: The genomic signalling pathway is characterised by a change in gene expression which is mediated by direct interactions with the DNA.

Non-genomic signalling: The non-genomic signalling pathway is characterised by rapid cellular responses mediated by secondary messenger proteins.

Model: refers to the protein structure used for ligand docking obtained after the required processing and preparation of the published crystal structure is completed. The model also still contains the co-crystallised ligand.

Additivity: In this thesis additivity describes the case in which xenoestrogens act together to produce effects without enhancing or diminishing each other's action.

Nomenclature

The notation for ring designation and numbering substituent positions is illustrated in Figure A. The ring designation is often used in reference to other ligands that do not have this structure to describe the type of interactions with the receptor. Note that the ring designation given in Figure A for genistein does not follow the common convention for flavone ring designation, but is used in the ER literature.

The binding cavity of the ERs is also described as having an A-ring end and a D-ring end, in reference to the usual orientation of E2 in the binding cavity. The A-ring end is the Glu/Arg/water molecule end. The D-ring end is the His end.

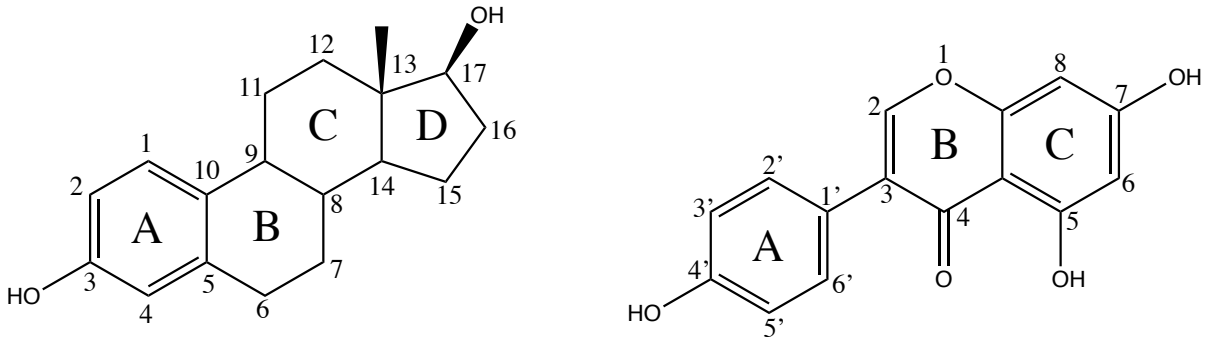


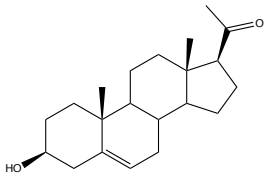
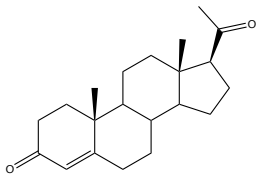
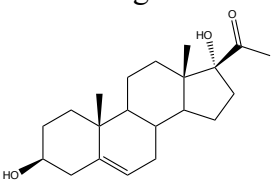
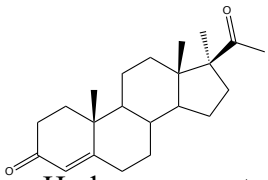
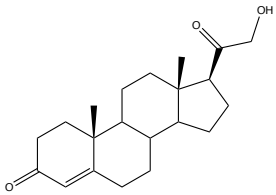
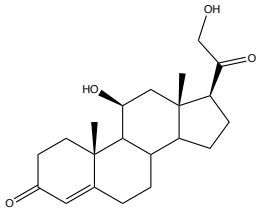
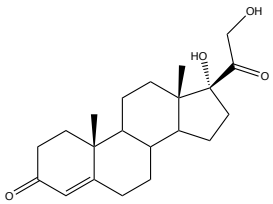
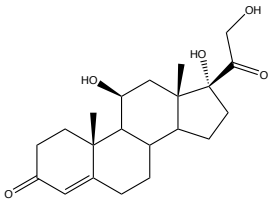
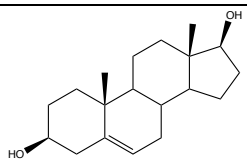
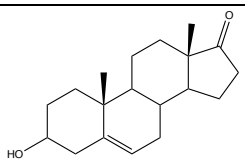
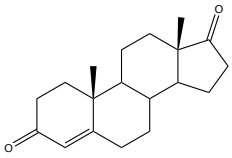
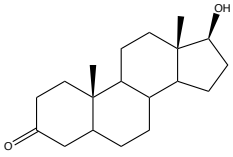
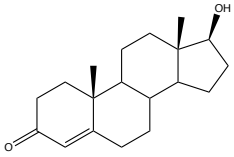
Figure A. Position numbering and ring notations for ligands, illustrated with E2 and genistein.

Chapter 1 Introduction

1.1 General Introduction to Hormones

A plethora of hormones regulates the body's functions including growth and development, metabolism, electrolyte balance and reproduction (Hiller-Sturmhofel, *et al.*, 1998). The endocrine system is a primary regulatory mechanism in the body. It synthesises and secretes hormones into the circulatory system and extracellular fluids in order to control and coordinate the function of distant organs, tissues and cells through receptor mediated responses. In general, there are 3 classes of hormone in mammals; steroid hormones (e.g. 17β-estradiol (E2)), protein and peptide hormones (e.g. insulin) and hormones derived from single amino acids (e.g. serotonin) (Leblebicioglu, *et al.*, 2013). Endogenous steroid hormones are derived from a common precursor, cholesterol. They include estrogens, androgens, corticosteroids and progestogens (Fig. 1.1). Steroid hormones act via their cognate nuclear receptor; for example, estrogens act via the estrogen receptors and testosterone via the androgen receptor. Nuclear receptors are located in the cytoplasm and/or the nucleus of the target cells and are ligand inducible transcription factors, meaning that when the endogenous hormone diffuses into the cell, it binds a specific receptor protein and initiates transcription of target genes (Enmark, *et al.*, 1999, Evans, 1988, Jensen, *et al.*, 1972, Nilsson, *et al.*, 2002, Nilsson, *et al.*, 2001). Testosterone and E2 are two key steroid hormones important in the development of the fetus, particularly in determining whether the fetus develops male or female reproductive organs (Blaschko, *et al.*, 2012, Jost, 1965, Mello, *et al.*, 2005, Nef, *et al.*, 2000). E2 is essential for developing primary and secondary sex characteristics in females, maintaining the female reproductive cycle, and in pregnancy (Zhu, *et al.*, 1998). Progesterone is another endogenous steroid hormone that is involved in the complex regulation of normal female reproductive function. Progesterone has major physiological roles in the uterus, ovary, mammary gland and brain, and functions via its cognate nuclear receptor, the progesterone receptor (PR) (Graham, *et al.*, 1997). The ratio of steroid hormones is crucial. If there are more estrogens present in a male fetus the formation of the sex organs is affected. This can cause birth defects such as

hypospadias where the urethra forms abnormally, and cryptorchidism where one or both of the testes fail to descend (Aksglaede*, et al.*, 2006).

Progestogens	 Pregnenolone  Progesterone  17α–Hydroxypregnenelone  17α– Hydroxyprogesterone
Corticosteroids	 11-Deoxycorticosterone  Corticosterone  Cortisol  11-Deoxycortisol
Androgens	 Androstenediol  Dehydroepiandrostenedione  Androstenedione  5α–Dihydrotesterone  Testosterone

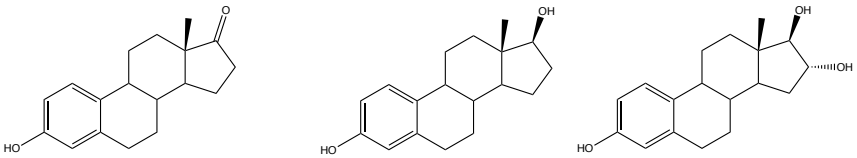
Estrogens	 Estrone E2 Estriol

Figure 1.1: The key progestogens, corticosteroids, androgens and estrogens.

There are two estrogen receptor (ER) isoforms, ERα and ERβ, found in different amounts in different tissues, which respond differently to the estrogens. Therefore, the distribution of ER is important when assessing its role in each target tissue. The classical ERα targets include the uterus, mammary gland, placenta, liver, central nervous system, cardiovascular system and bones (Couse*, et al.*, 1999, Couse*, et al.*, 1997, Zhang*, et al.*, 2003). ERα responds to E2 with an increase in transcription of estrogen responsive genes, aiding in glucose metabolism and mating behaviour (Cross*, et al.*, 1999, Laredo*, et al.*, 2014, Mauvais-Jarvis*, et al.*, 2013). The ERβ target tissues include the prostate, testes, ovaries, pineal gland, thyroid, parathyroid, adrenals, pancreas, gallbladder, skin, urinary tract, lymphoid and erythroid tissues, lung, thymus, spleen, and localised areas of the brain (Couse*, et al.*, 1997, Gustafsson, 1999). ERβ is essential for normal ovulation efficiency and immune responses but not for female or male sexual differentiation, fertility or lactation. ERα is often undetectable in ERβ dominant tissues (Weiser*, et al.*, 2008, Ying*, et al.*, 2000); however, ERβ is present in ERα dominant tissues and acts to control the expression of ERα, reducing the potency of estrogens acting via ERα (Hall*, et al.*, 1999, Henley*, et al.*, 2006, Lindberg*, et al.*, 2003). There are 1069 estrogen responsive human genes corresponding to 1051 unique target promoters, according to the Knowledge Base for Estrogen Responsive Genes (KBERG) (Tang*, et al.*, 2007). Many of these genes share ER isoform commonality, meaning both ER isoforms can induce gene transcription via the sites.

1.2 Estrogens

There are three endogenous estrogens, E2, estrone and estriol. They were discovered in the 1920's and 1930's by Adolf Butenandt and Edward Adelbert Doisy. Butenandt extracted them from horse urine and in 1939 won The Nobel Prize in Chemistry for his work on sex hormones (Tata, 2005). E2 cannot be stored in tissues, so is synthesised when required (Hanukoglu, 1992). The role and presence of estrone, E2 and estriol in the body differ greatly depending on age, gender, stage of development and whether a woman is pregnant (Green*, et al.*, 1987). The use of estrogens has had a huge impact, especially in human health and reproduction, for example the use of synthetic estrogens in contraception (Dhont, 2010).

Total serum (free and protein bound) E2 levels vary substantially over the development and lifetime of a human and are different between males and females. Graham, (2012) reviewed the literature and demonstrated these differences (Fig. 1.2). These data highlight the dramatic increase in E2 levels at birth and the rapid decline in the first few days of life. Subsequently, the levels decline in post-menopausal women to levels similar to those of children aged between 1-5 years.

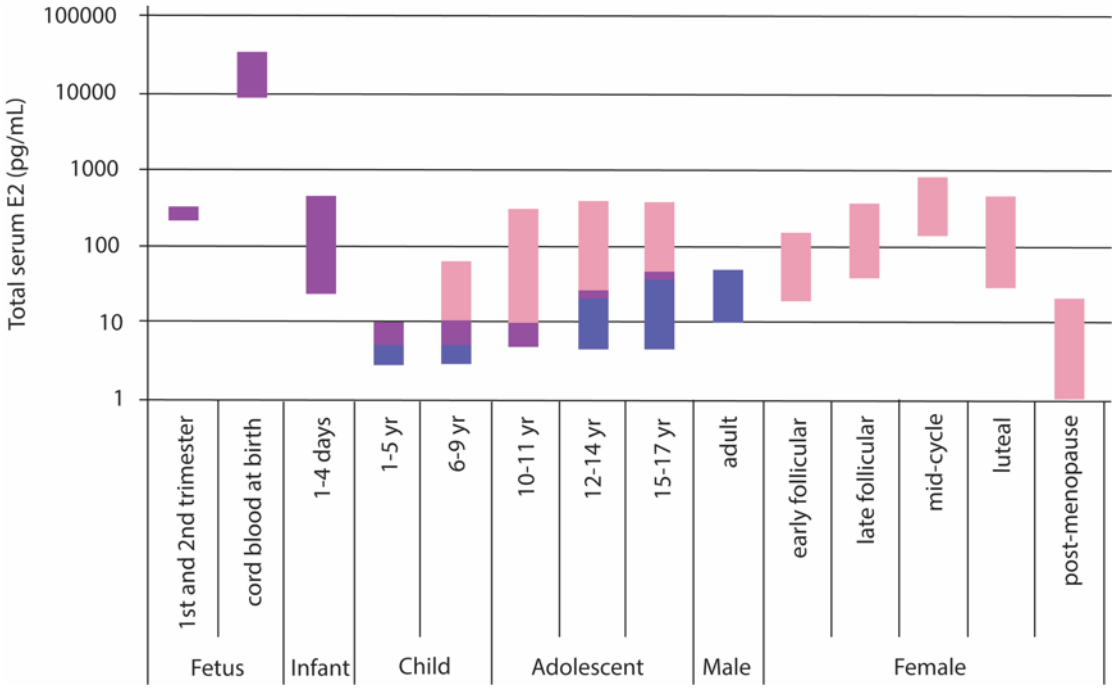


Figure 1.2: Human total (free + protein bound) serum E2 concentrations (Fowler, *et al.*, 2011, Graham, 2012, Shutt, *et al.*, 1974, Styne, 2004). Blue bars indicate male levels, pink bars indicate female levels. The overlap of male and female levels appears grey (graph from Graham, 2012 with permission).

There are many extensive reviews on the physiological effects of estrogens in the body in both males and females (Kuiper, *et al.*, 1998, Nilsson, *et al.*, 2002). To summarise, in females they are responsible for stimulating pubescent growth characteristics such as breasts, wider pelvis, fatty tissue around the buttocks and hips. In adults, they regulate the menstrual cycle, vaginal secretions and prepare the body for pregnancy by thickening the uterine lining. In males, E2 is mainly produced in the testes but also in the adrenal and pituitary glands. They promote the growth of the penis and development of the testicles, stimulate growth of facial and body hair, deepening of the voice and growth and maturation of sperm. High E2 levels are also linked to high libidos in men. In both males and females, they are important in cardiovascular function and the nervous system e.g. learning, memory, awareness, fine motor skills, temperature regulation, mood and reproductive functions.

Estrogens are synthesised, when required, from the initial conversion of cholesterol to androstenedione. This can be directly converted into estrone or E2 via testosterone, catalysed by the enzyme aromatase. Estrone and E2 circulate around the body bound

to plasma-steroid binding hormones (known as steroid hormone binding globulin; SHBG) and albumin (Nagel*, et al.*, 2004). Alternatively, E2 is also present as water soluble sulfate and glucuronide conjugates. E2 is metabolised via either hydroxylation by cytochrome P450 enzymes or via conjugation by estrogen sulfotrasnferases (sulfation) and UDP-glucuronyltransferases (glucuronidation). E2 is also dehydrogenated into the much less potent estrone. This is then stored in the body as estrone sulfate and is converted back into E2 when required (Liu, 2009). The unbound estrogens passively diffuse in and out of cells and only a small fraction (approximately 2%) of these unbound estrogens is responsible for their biological activity (Nagel*, et al.*, 2004).

1.2.1. 17β-Estradiol

Estradiol is the primary and most potent of the endogenous female estrogen hormones. It exists as 2 isomers, 17α–estradiol and 17β–estradiol or E2, with the only distinguishing feature being the stereochemistry of carbon atom 17 (Dykens*, et al.*, 2005, Littlefield*, et al.*, 1990). 17α–Estradiol is not found in high concentrations and, compared with E2 has little to no estrogenic activity in ERα and ERβ; 58% and 11% compared with E2, respectively (Moos*, et al.*, 2008). E2 is the dominant form of estradiol and is known to be responsible for the majority of estrogen action. It is synthesised from testosterone in many tissues and is responsible for gender determination in a fetus, the onset of puberty and maintaining female secondary sex characteristics including the reproductive cycle(Kleine*, et al.*, 2016). E2 is also present in males but at much lower concentrations than in females of reproductive age; akin to concentrations found in prepubescent girls and post-menopausal women (Bergman*, et al.*, 2012). Women of child bearing age have much higher concentrations of E2, which fluctuate during the menstrual cycle. During the first part of the menstrual cycle, levels of E2 rise slowly then peak sharply, initiating ovulation (Fig. 1.3) (Klump*, et al.*, 2013).

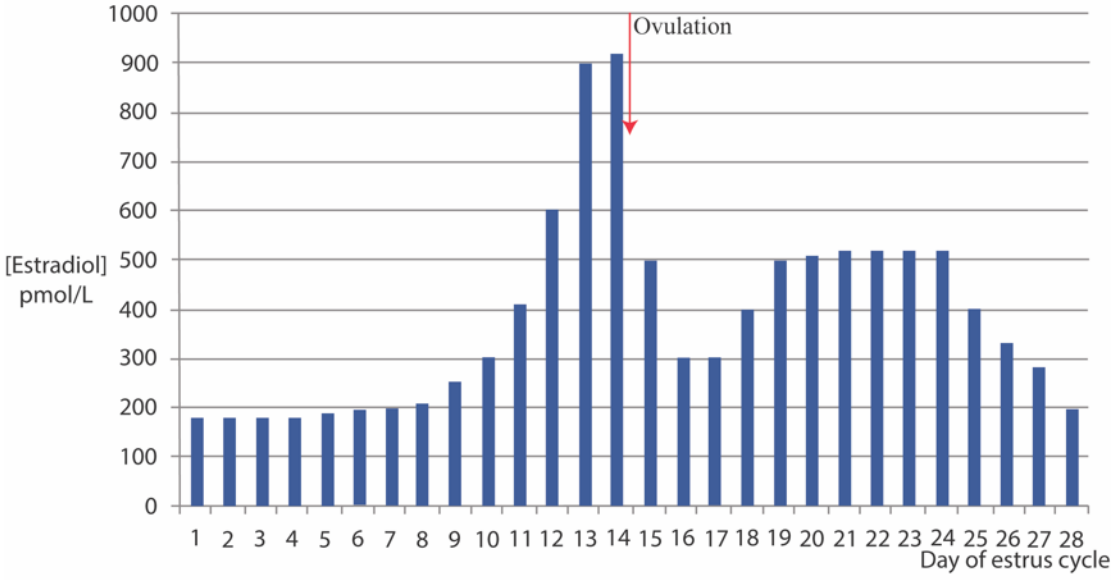


Figure 1.3: Blood levels of estradiol in women showing the large changes during the estrus cycle – ovulation occurs on day 13 or 14 (data from http://commons.wikimedia.org/wiki/File:Estradiol_during_menstrual_cycle.png)

1.2.2. Estrone

Estrone is present at the lowest plasma concentration of the endogenous estrogens. Estrone and its sulfonated conjugated form, estrone sulfate, are most commonly found in menopausal women as their main form of estrogen. Most of the naturally occurring estrogen in post-menopausal women is synthesised in the adrenal cortex and other peripheral tissues from androstenedione (Kleine*, et al.*, 2016). Estrone is a metabolite of E2 (Fig. 1.4), which is converted to E2, the more active estrogen, when needed (Hanukoglu, 1992). It is secreted by the ovaries along with E2 in women with normal menstrual cycles and by the placenta in pregnant women (Kleine*, et al.*, 2016).

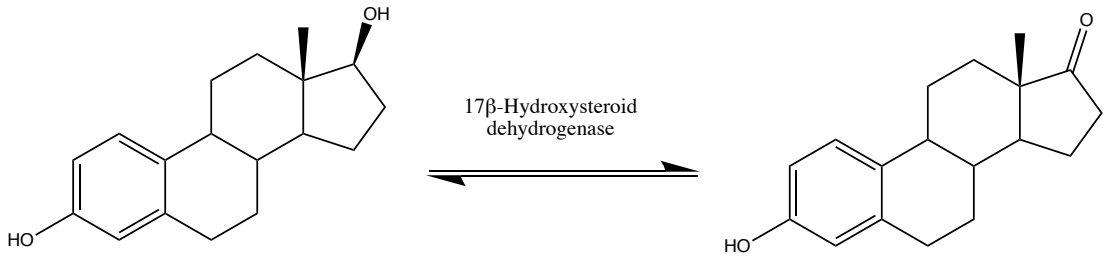


Figure 1.4: E2 is metabolised to estrone via 17β-hydroxysteroid dehydrogenase. This reaction is reversible.

1.2.3. Estriol

Estriol is made in significant amounts by the placenta during pregnancy. When isolated from human placenta in 1931, the concentration of estriol found was much higher than the concentrations of estrone or E2. Studies show that although estriol levels present in pregnant women are high compared with non-pregnant and post-menopausal women, estriol is not as potent as E2 (Heller, 1940). Interestingly, estriol was originally used to treat undesirable symptoms of menopause because it had been observed that estriol is present in the blood of women of reproductive age but is rarely found in the blood of perimenopausal women (Merrill, 1958). It has also been shown that estriol alone, acts as an ER agonist, but in the presence of E2 it acts as an antagonist (Melamed*, et al.*, 1997). Estriol is a metabolite of E2; however, its biochemical function is not as well understood as other estrogens. E2 is converted to estriol via a reaction with cytochrome P450 (Fig. 1.5) (Thomas*, et al.*, 2013).

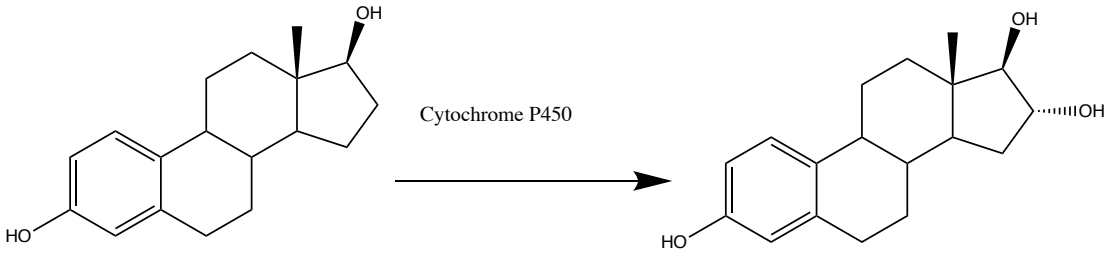


Figure 1.5: E2 is metabolised to estriol via cytochromes P450.

1.3 Estrogen Receptors

The ERs are ligand inducible transcription factors that mediate most of the biological effects of estrogens in the body (Evans, 1988). There are 2 classes of ERs, (i) nuclear ERs (e.g. ERα and ERβ) which belong to the nuclear receptor family and (ii) membrane ERs (e.g. GPER) (Hammes*, et al.*, 2007) which are mainly G protein-coupled receptors (Filardo*, et al.*, 2000, Revankar*, et al.*, 2005). The membrane ERs are cell surface receptors that are activated by endogenous estrogens (Soltysik*, et al.*, 2013). Current information is limited on the actions of these receptors; therefore, ER will refer to nuclear ERs from here on unless otherwise stated. There are two genetically distinct forms of ER; ERα and ERβ. The ERα gene ESR1 is located on chromosome 6q25.1 and the ERβ gene ESR2 is located on chromosome 14q23.2 (Ariazi*, et al.*, 2006, Ascenzi*, et al.*, 2006). ERα consists of 595 amino acids and has a molecular weight of 66 kDa. Comparatively, ERβ consists of 530 amino acids and has

a molecular weight of 55 kDa. ERα was first identified in 1958, and cloned in 1986 (Green, *et al.*, 1986). ERβ was not identified until 1996 (Mosselman, *et al.*, 1996).

Both ERα and ERβ have 6 functional domains (A-F) which have varying amino acid commonality between domains (Couse, *et al.*, 1997, Hall, *et al.*, 2005, Kumar, *et al.*, 2011). These are the N-terminal or A/B domain, the DNA binding domain (DBD), the ligand binding domain (LBD), the hinge region (D) and the F or c-terminal region (Fig. 1.6) (Kumar, *et al.*, 2011). Although the primary structure of these domains is well defined, there is limited understanding of the structural dynamics of ERs, including intra- and intermolecular communication under the influence of various associated coregulatory proteins and post translational modifications. A major obstacle to understanding the structural dynamics of ERs has been the complete structural characterisation of the ERs. Some regions (i.e. the DNA and ligand binding domains) have been crystallised and reside in a well ordered state (Brzozowski, *et al.*, 1997, Huang, *et al.*, 2013, Johnson, *et al.*, 2012, Nettles, *et al.*, 2007, Simons, *et al.*, 2013, Wu, *et al.*, 2005); however, other regions reside in an intrinsically disordered (ID) state, making any x-ray crystallography techniques almost impossible (Dahlmanwright, *et al.*, 1995, Kumar, *et al.*, 2003, McEwan, *et al.*, 1996, Shen, *et al.*, 1996, Simons, *et al.*, 2013, Warnmark, *et al.*, 2001).

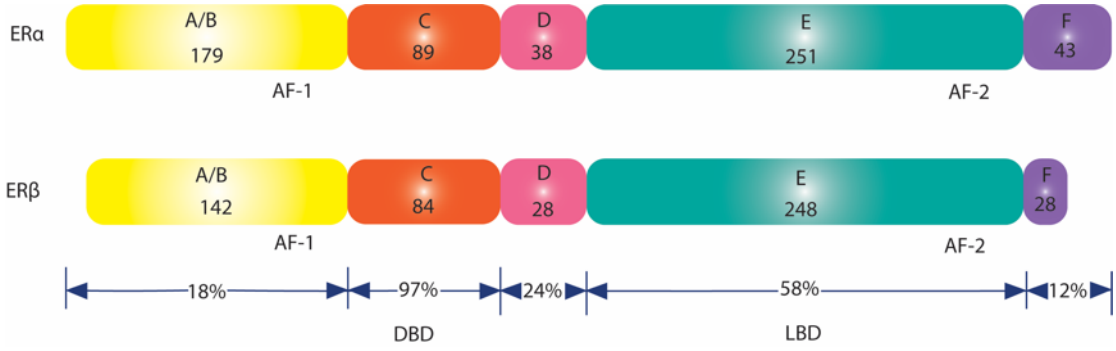


Figure 1.6: Schematic representation of estrogen receptor protein structural domains. Within each segment, the number of amino acid residues is given. The % homology is indicated below each domain (from Graham, 2012 with permission).

1.3.1. ER Structure

1.3.1.1. The N-terminal Domain

The N-terminal domain or the A/B region of ERs do not have well-defined tertiary structure and exist in an ID state (Ascenzi*, et al.*, 2006). One of the reasons for this is the ID region appears to help promote molecular recognition by providing surfaces capable of binding specific target molecules. This has been confirmed by circular dichroism, which showed 67% of the ERα N-terminal domain contains a random coil conformation. Comparatively, ERβ has over 80% random coil conformation in its N-terminal domain (Combet*, et al.*, 2000, Warnmark*, et al.*, 2001). It is thought that the high percentage of random coils allows for random sampling of the environment and facilitates appropriate concentration and affinity of the binding partners and proteins (Kumar*, et al.*, 2003). Although the N-terminal region is not well defined structurally, it is thought to be involved in transcriptional regulation and has the activation function (AF)-1 functional region. AF-1 activates target genes by interacting with components of the transcriptional machinery that regulate gene transcription (Enmark*, et al.*, 1999). It is constitutively active e.g. ligand independent. Among the ER transcription machinery, it is also regulated by insulin like growth factors and epidermal growth factors (Cozzini*, et al.*, 2004, Hall*, et al.*, 2005). This region exhibits on of the most distinctive differences between ERα and ERβ; with the ERβ region being significantly shorter (Kumar*, et al.*, 2011). Interestingly, it is thought that the AF-1 region on the ERβ isoform is a repressor region rather than an activation region as the name suggests (Delaunay*, et al.*, 2000, Hall*, et al.*, 1999).

1.3.1.2. The DBD

The DBD is the most similar region of the two ER isoforms, with 95% amino acid commonality (Cowley*, et al.*, 1997). This region binds with a high affinity to specific DNA sequences in target gene promoter regions known as estrogen responsive elements (ERE). The ERE is composed of a palindromic hexanucleotide 5' AGGTCAnnnTGACCT3' (where n=any base) (Kleinhitpass*, et al.*, 1989, Roche*, et al.*, 1992, Wood*, et al.*, 2001). Unsurprisingly, due to their high amino acid commonality, both ER isoforms share a high proportion of EREs (Kumar*, et al.*, 2011). The DNA binding region plays an important role in receptor dimerization, providing the surfaces for the head to head dimerization of two receptor molecules. It also dictates the binding affinity of the ER and modulates the recruitment of

coregulator proteins (Ascenzi*, et al.*, 2006, Enmark*, et al.*, 1999). The binding of the dimerised ER at EREs allows recruitment of transcriptional machinery and specific coregulatory proteins to transcribe the specific ER target genes. It comprises P box, D box, and zinc finger subdomains. The zinc finger subdomains are comprised of 8 cysteine residues that coordinate with two Zn^{2+} ions. The P box actively interacts with the ERE nucleotides and the D box is present at the dimerization interface (Figs. 1.7 and 1.8) (Koide*, et al.*, 2007, Luisi*, et al.*, 1991, Montano*, et al.*, 1995).

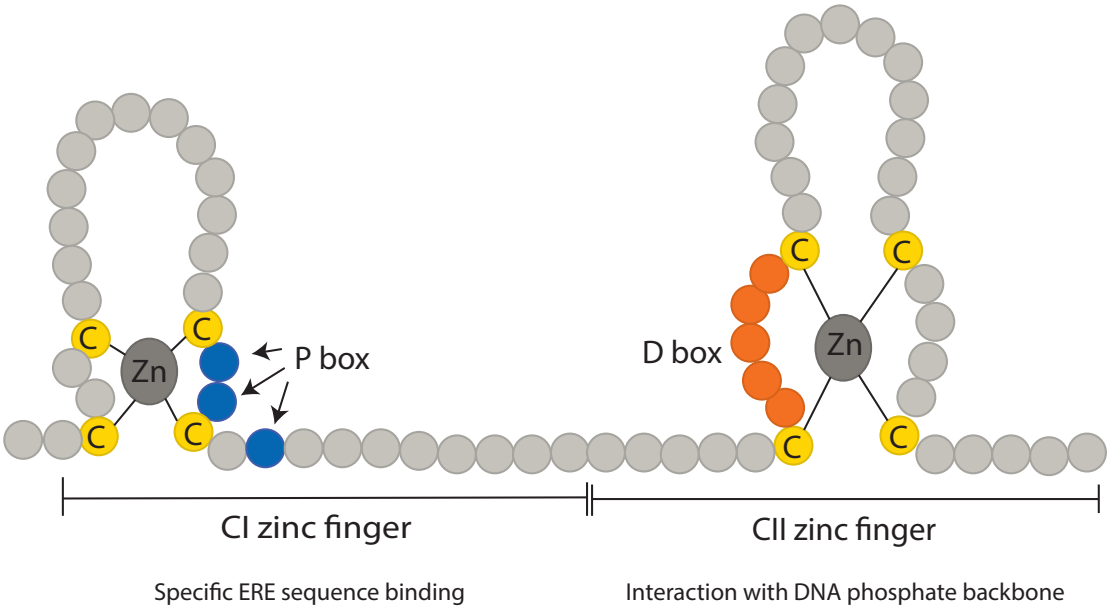


Figure 1.7: Schematic diagram of type II zinc finger DNA binding domain of the ERs. Each circle represents one amino acid. The CI zinc finger interacts specifically with 5 base pairs of DNA and determines the DNA sequences recognized by the ER. The blue amino acids in the knuckle of the CI zine finger are in the P box and allow recognition of the ERE. The orange amino acids within the knuckle of the CII zinc finger constitute the D box, which is important for dimerization and contacts with the DNA phosphate backbone (website no longer available).

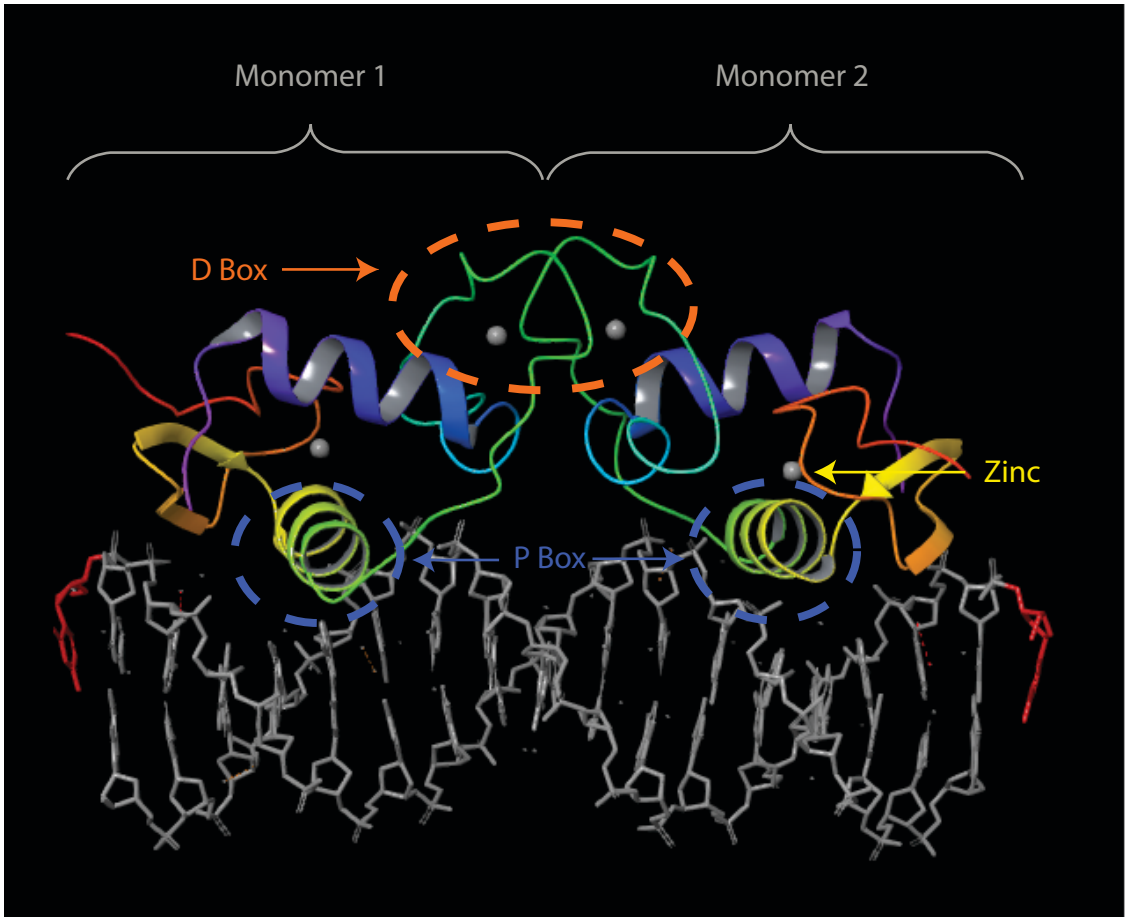


Figure 1.8: Tertiary structure of the ERα DBD dimer complexed with DNA and zinc (shown is space filling mode). Atom co-ordinates from PDB entry 1HCQ. Image produced in Schrödinger Suite 2017 Maestro.

1.3.1.3. The D Domain

The D domain, or the hinge region, has not been well characterised but is thought to contribute to the flexibility of the receptor structure, as it is located between two highly conservative domains of the receptor molecule, the DBD and ligand binding domain. There is only 36% amino acid commonality between the two ER isoforms, and this is thought to influence the DNA binding properties of individual receptors, while serving as an anchor for certain corepressor proteins (Ascenzi*, et al.*, 2006, Enmark*, et al.*, 1999, Kuiper*, et al.*, 1996).

1.3.1.4. The LBD

The LBD is the most studied region of the ERs. The LBD is composed of two binding sites, the ligand binding cleft (LBC), where E2 generally binds, and activation function (AF)-2 where coregulator proteins interact (Fig. 1.9) (Zhang, *et al.*, 2003). The LBD has been well characterised individually, but not in the context of the whole receptor. It has been the source of many structural studies which have led to a significant proportion of current knowledge about the ERs (Blizzard, *et al.*, 2005, Brzozowski, *et al.*, 1997, Manas, *et al.*, 2004, Mocklinghoff, *et al.*, 2010, Shiau, *et al.*, 1998, Tanenbaum, *et al.*, 1998).

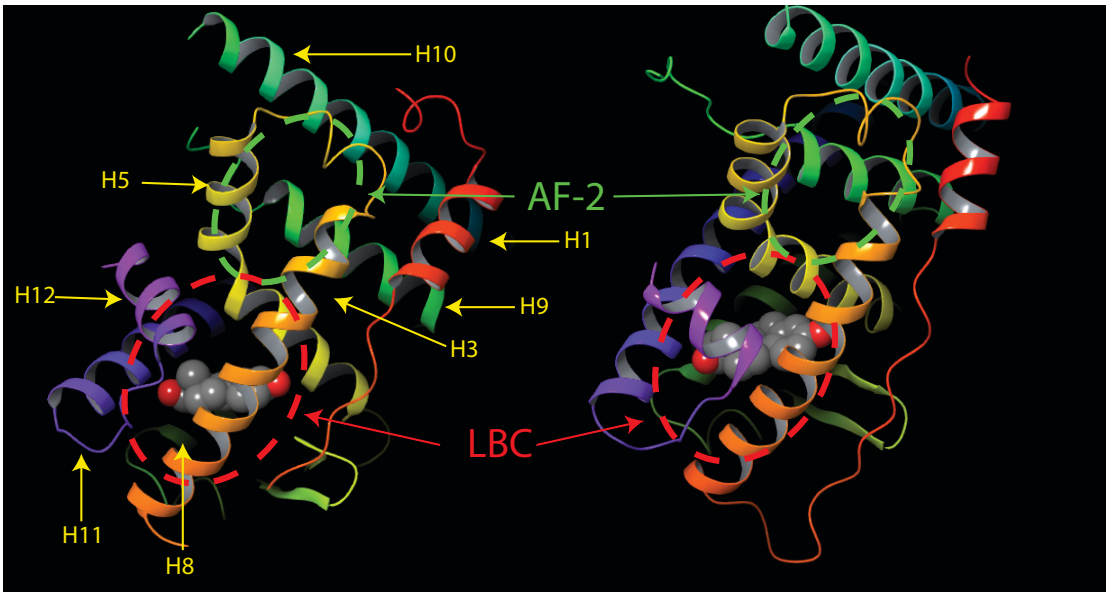


Figure 1.9: Tertiary structure of the ERα LBD with E2 (left) and ERβ LBD with E2 (right). Ribbons illustrate the α-helix protein structure. Thin lines illustrate flexible loops which are disordered regions connecting two ordered regions. Ribbons with arrows illustrate β sheet structure. The LBC (red) and AF-2 (green) are highlighted by dashed circles. Atom co-ordinates from PDB entry 1ERE (ERα) and 3OLS (ERβ). Image produced in Schrödinger Suite 2017 Maestro.

The structure of the ERs was first shown by Brzozowski (1997) and has been extensively reviewed since then; however, there has been no substantial new information on ERs since then but rather new interpretations of the structural dynamics and importance of the binding sites of the ERs(Ye, *et al.*, 2018). The LBD has a globular structure that houses 11 α helices (Brzozowski, *et al.*, 1997, Wu, *et al.*, 2005) that are spatially organised in a 3-layered structure with helices 4, 5, 6, 8 and 9

lining up on one side of helices 1 and 3 with helices 7, 10 and 11 on the other side. Additionally, Helix 12 resides in a perpendicular orientation depending on the ligand bound to the LBC (Fig.1.12) (Jordan*, et al.*, 1985, Moore*, et al.*, 2010, Robertson, 2004). For example, helix 12 will cap the LBC when an agonist is bound to the LBC; however, it will reside in a slightly different orientation, often blocking the AF-2 site, when an antagonist is bound (Fratev, 2015). The agonist orientation of helix 12 allows for E2 to be buried in a highly hydrophobic environment (Brzozowski*, et al.*, 1997). E2 interacts via its hydroxyl groups at positions 3 and 17, which plays a crucial role in orienting the hormone in the hydrophobic pocket (Moore*, et al.*, 2010). The A and D rings are hydrogen bonded to Glu353 (305 ERβ), Arg394 (346 ERβ), water and His524 (475 in ERβ) (Fig. 1.10) (Brzozowski*, et al.*, 1997, Jordan, 2003).

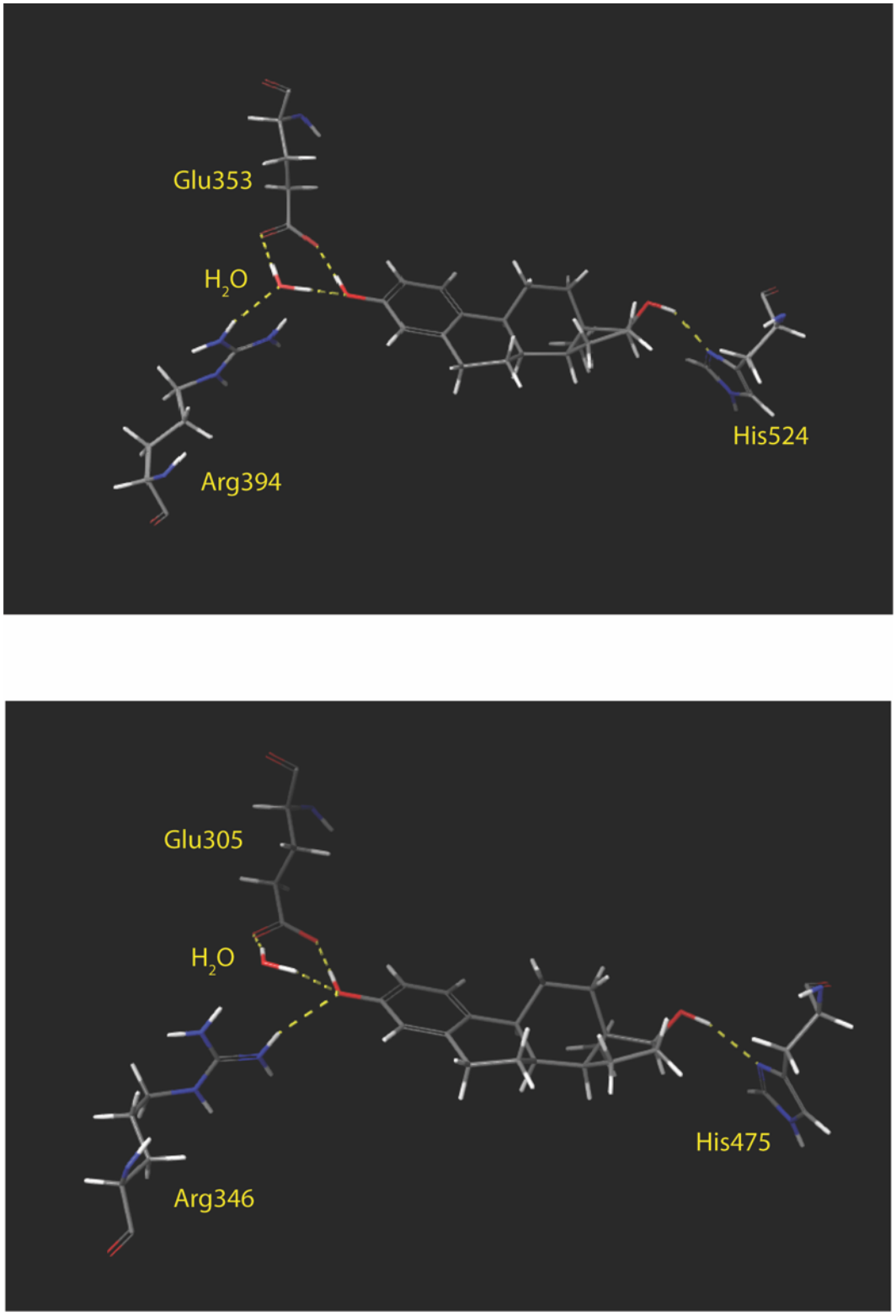


Figure 1.10: Hydrogen bonding network of E2 in ERα (i) and ERβ (ii). Atom co-ordinates from PDB entry 1ERE and 3OLS. Image produced in Schrödinger Suite 2017 Maestro.

The AF-2 site

The AF-2 site binds both the heat shock protein (e.g. when the ER is in its inactive state) and coregulatory proteins (e.g. coactivators and corepressor) (Huang, *et al.*, 2013). Like the LBC, the AF-2 site is a highly hydrophobic groove and is formed by helices 3, 4, 5 and 12 and the turn between 3 and 4 (Shiau, *et al.*, 1998). Coregulatory proteins interact via a highly conserved LxxLL motif (where L=leucine and x=any other amino acid). The AF-2 site also functions cooperatively with the AF-1 region in the ERα isoform, synergistically activating the ER and thus transcription (Metivier, *et al.*, 2001). The AF-2 site is strictly ligand dependent (Cozzini, *et al.*, 2004), requiring ligand binding at the LBC, which causes a conformational change exposing the AF-2 site.

Partial and full antagonists induce conformations at the AF-2 region that are distinct from that observed in the presence of a pure agonist (Pike, *et al.*, 2001). Classic examples are the breast cancer drugs raloxifene and tamoxifen, and the tamoxifen metabolite 4-hydroxytamoxifen (4-OHT) which both have structural similarities to E2 (Fig. 1.11).

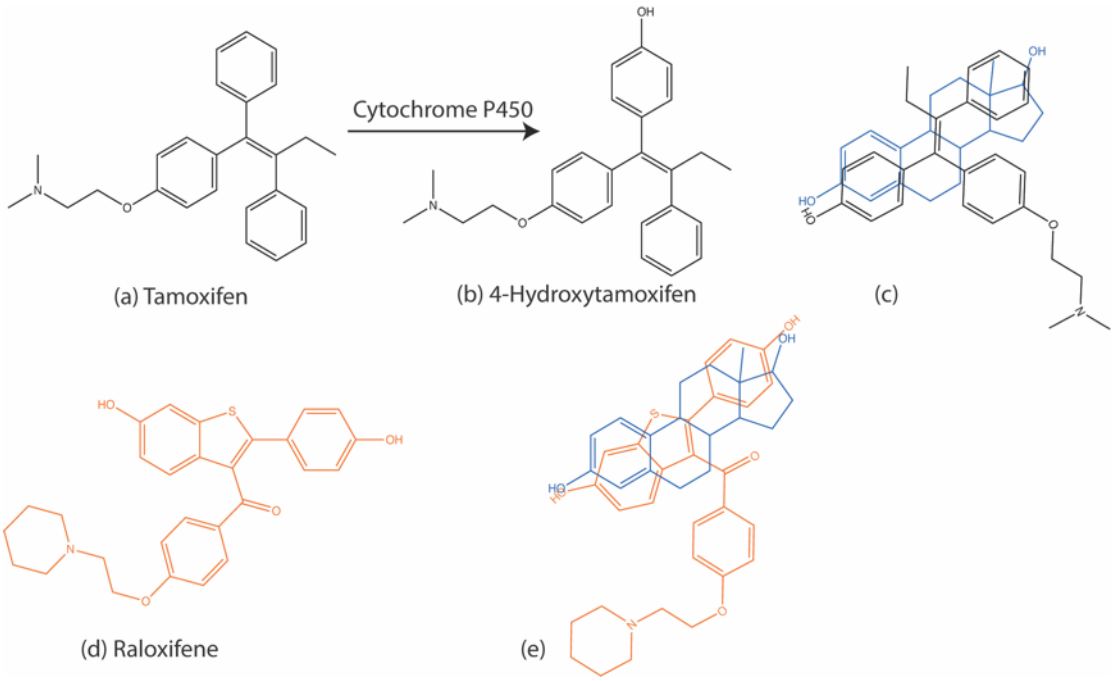


Figure 1.11: Structures of (a) tamoxifen, (b) 4-hydroxytamoxifen (4-OHT) and (d) raloxifene overlayed with E2 (blue) (c and e).

The binding of these drugs is accompanied by large structural changes in the tertiary structure of both ER isoforms (Brzozowski*, et al.*, 1997, Pike*, et al.*, 2001, Shiau*, et al.*, 1998). The large piperidine extensions of the ligand provoke steric clashes that prevent H12 from adopting its characteristic conformation and thus formation of the AF-2 site. Instead, H12 lies in the would-be AF-2 site via a LxxML motif (where L=leucine, M=methionine and x=any other amino acid) (Fig. 1.12) (Pike*, et al.*, 2001).

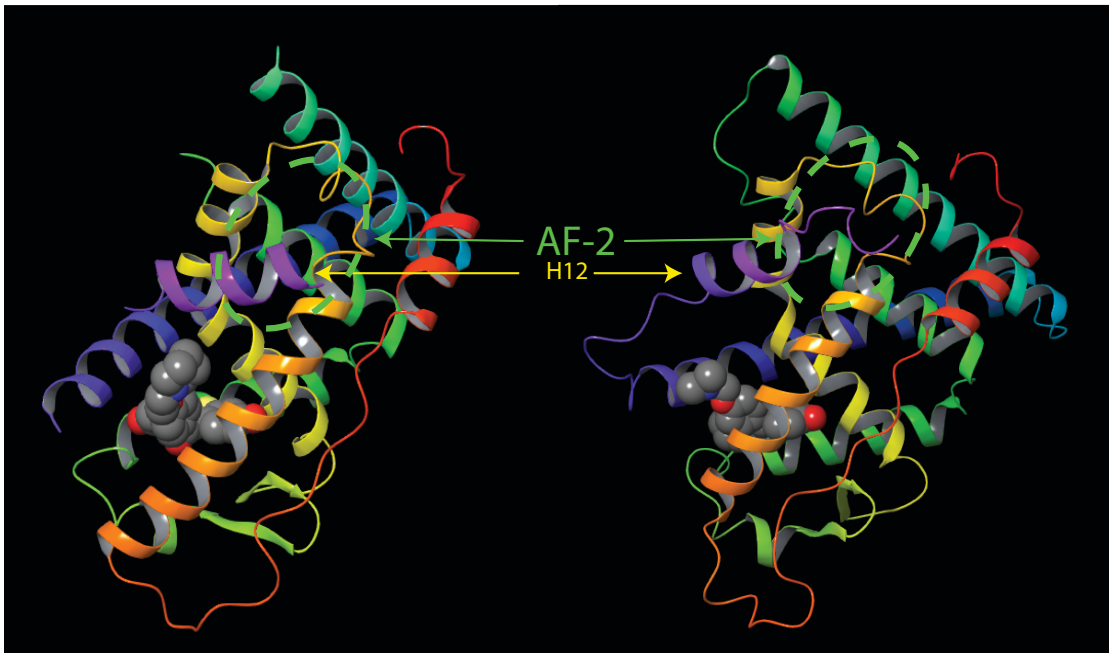


Figure 1.12: Tertiary structure of ERα complexed with raloxifene (left) and 4-hydroxytamoxifen. The AF-2 site is occupied with H12. Atom co-ordinates from PDB entry 1ERR (raloxifene) and 3ERT (4-hydroxytamoxifen). Image produced in Schrödinger Suite 2017 Maestro.

Interestingly, ERs associate with corepressors far more frequently than coactivators. The ERs associate with corepressor proteins that silence their activity in the absence of ligands, and activation therefore, involves displacement of the associated corepressors by the coactivators, an event that permits the functional interaction of the receptor with the cellular transcriptional machinery. More recently coregulator proteins have been studied, mainly through the use of peptides which highlight their key interactions with the ERs at AF-2. These are Lys362 and Glu542 (ERα) (Sun*, et al.*, 2011) and Lys314 and Glu493 (ERβ) (Wang*, et al.*, 2006). These amino acids are known as capping molecules and are what initially attracts the coregulatory proteins to interact with the AF-2. The coregulatory proteins have multiple (up to three)

LxxLL (L=leucine and x= any other amino acid residue) motifs, where the carbonyl group on the amino acid backbone hydrogen bonds to the lysine and glutamic acid in the AF-2 domain, along with other key amino acid residues (Nolte*, et al.*, 1998, Shiau*, et al.*, 1998). These include van der Waals interactions with Leu310, Phe319, Leu324, Gln327, Val328, Leu331, Lue332, Leu490 and Met494 (Fig. 1.13) (Nolte*, et al.*, 1998). Current structural information on these interactions is limited due to the use of peptides rather than whole protein. Coregulatory proteins are distinctly larger than ERs, with their size ranging upwards of 160 kDa. Therefore, any complete structural information is almost impossible with current techniques.

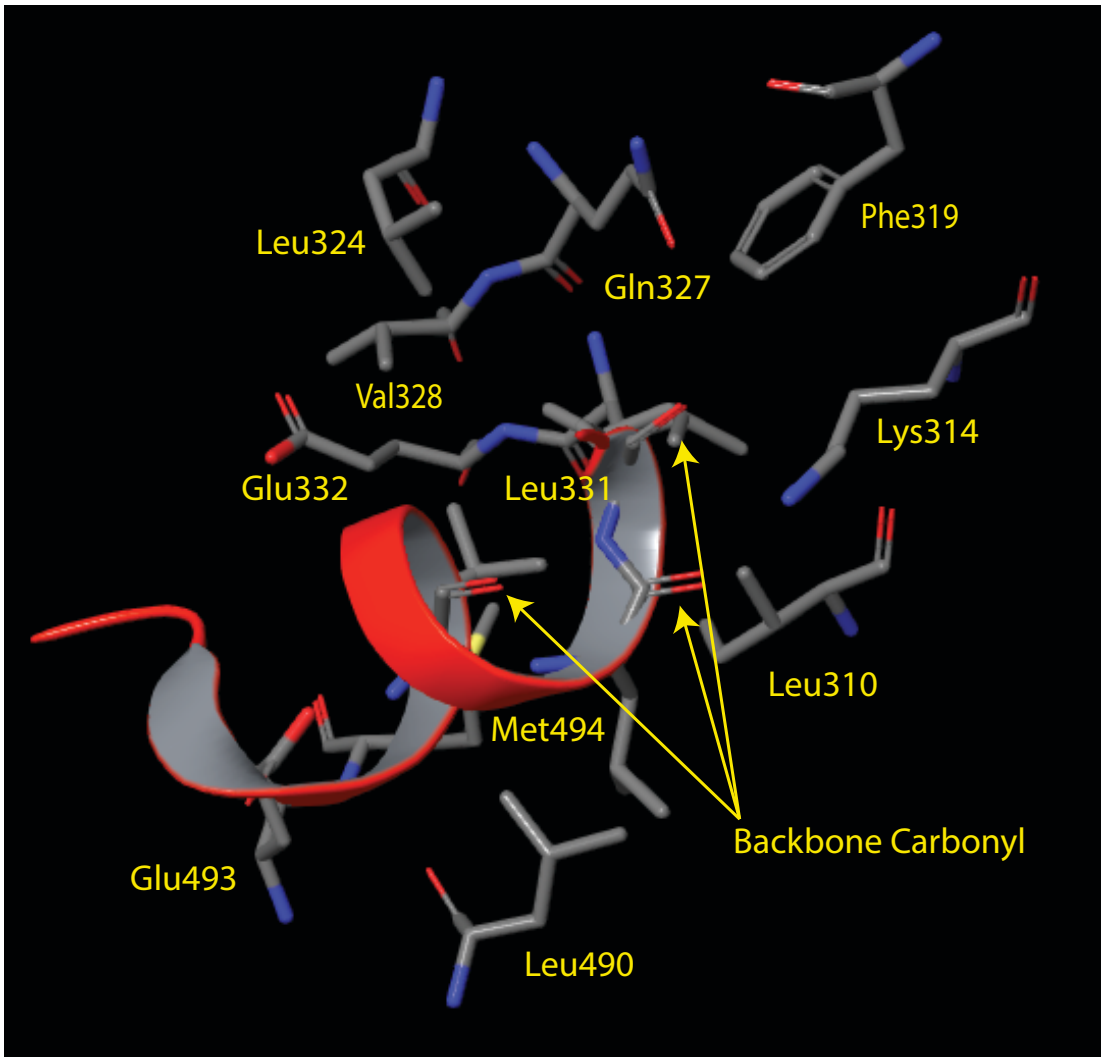


Figure 1.13:Tertiary structure of the ERβAF-2 site complexed with steroid receptor coactivator (SRC) 1 peptide (red). Key amino acid interactions are highlighted. Atom co-ordinates from PDB entry 3OLS. Image produced in Schrödinger Suite 2017 Maestro.

However, over the last decade it has become clear the AF-2 binds more widely than first anticipated. Interestingly, Wang and colleagues, surprisingly, found 4-OHT can displace H12 in the initial antagonist conformation induced by 4-OHT binding at the LBC and bind itself to the AF-2 site, suggesting a dual mechanism of action for the highly used breast cancer therapeutic (Fig. 1.14) (Wang*, et al.*, 2006). Although this is still heavily debated, this possibility of small molecule binding may provide new insights into AF-2 interactions; this interaction could even suggest a new possible mechanism of action when it comes to the ERs LBD.

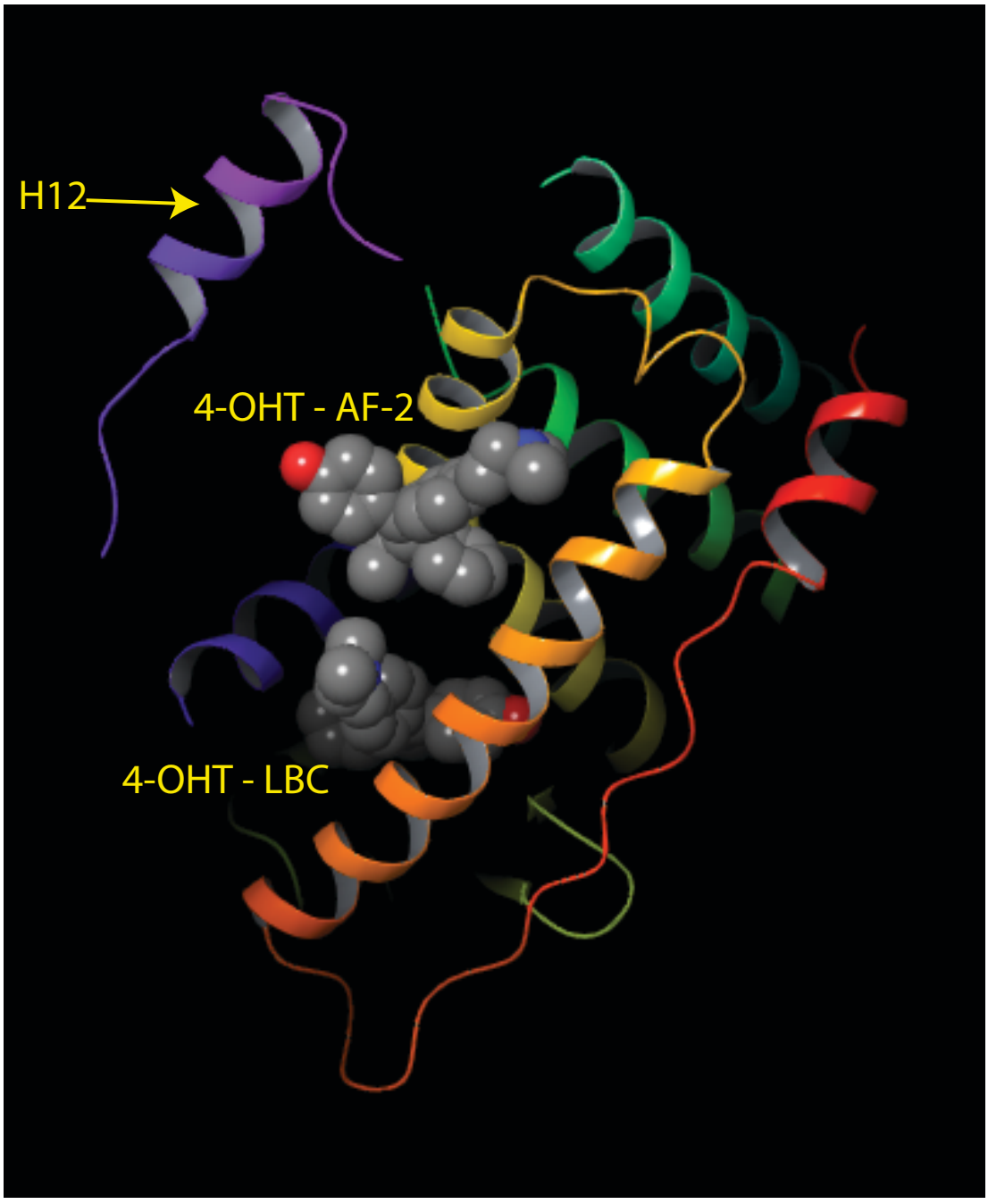


Figure 1.14: Tertiary structure of ERβ complexed with 4-hydroxytamoxifen (4-OHT) at both the LBC and AF-2. Atom co-ordinates from PDB entry 2FSZ. Image produced in Schrödinger Suite 2017 Maestro.

1.3.1.5. The F Region

The F region or C-terminus region contains 42 amino acid residues and is known to impact homo-and hetero-dimerisation (Montano*, et al.*, 1995). It also aids in the binding of the heat shock protein when the ERs are in an inactive state, thus modulates ligand mediated transcription. In addition, in an unliganded state, chaperone proteins interact with the F region, opening the binding cavity and making it accessible to the ligand (Ascenzi*, et al.*, 2006, Yang*, et al.*, 2010).

Therefore, ERs behave dynamically such that their kinetic behaviour allows them to readily interact with DNA, ligands, coregulatory proteins and chromatin. Two ER isoforms exist and share distinct regions of sequence homology. They have both overlapping and unique functions which leads to complex mechanisms of action via a dynamic network of cell mediated responses (Ascenzi*, et al.*, 2006, Yang*, et al.*, 2010).

1.3.2. ER Function

The ER has been defined as a cell receptor with exclusive nuclear localisation and its activity was considered solely derived from its direct interaction between EREs and the E2-ER complex, leading to initiation of gene transcription. Over the last 10-15 years, knowledge of this classical genomic mechanism of action has been enriched by the discovery of other alternative modes of action by the E2-ER complex. The complexity of cell biology results in interactions at the DNA and crosstalk of different pathways, both initiated by E2-ER complex binding. These different modes of action can be divided into three sub-classes of ER action: classical genomic, non-classical genomic and non-genomic.

1.3.2.1. The Classical Genomic Pathway

The best understood classical genomic pathway is where the ER is bound to a heat shock protein (HSP), usually HSP90 (90 = 90 kDa), via the AF-2 site. When a ligand (e.g. E2) binds in the LBC, it causes a conformational change that releases the ER from the HSP and closing of helix 12 into the AF-2 site (Zilli*, et al.*, 2009). The receptor undergoes dimerisation and phosphorylation and translocates to the nucleus where it binds to the EREs on the DNA via the DNA binding domain (Ascenzi*, et al.*, 2006). The AF-1 and AF-2 mediate positive regulation of gene expression of ER. The two AF domains often act synergistically, although some gene promoters are activated

independently by either AF-1 or AF-2 (Benecke*, et al.*, 2000). The transcriptional activity of ER is enhanced by binding of coactivators such as SRC1, TIF2, AIB1, etc. (Kim*, et al.*, 2016). These proteins form large complexes that enhance ER driven transcription, including recruitment of histone acetyltransferases at the promoter site. Some coactivators also have enzymatic activities that post translationally modify the chromatin, initiating unwinding, thus exposing the transcription site to the transcription machinery (e.g. RNA polymerase) (Bulynko*, et al.*, 2011). Part of the complex formed at the EREs includes proteins which aid in ER degradation. E1, E2 and E3 ligases mark the ER for proteasomal degradation via the ubiquitin proteasome pathway, allowing for further access to the EREs via new E2-ER complexes (Zhou*, et al.*, 2014). The ER is then recruited to the proteasome where it is degraded (Fig. 1.15) (Lee*, et al.*, 2008, Zhou*, et al.*, 2014).

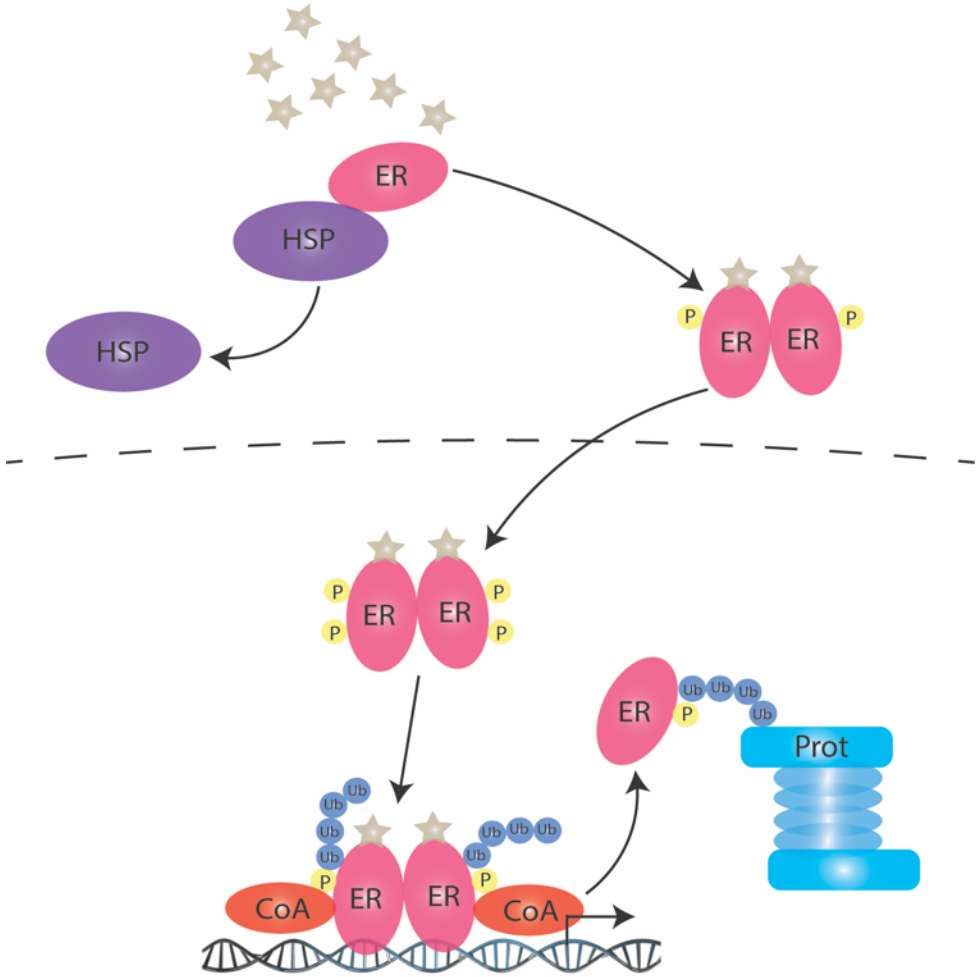


Figure 1.15: Illustration of the classical genomic signalling pathway. The ER monomer is activated upon E2 ( ) binding, facilitating dissociation from the heat shock protein (HSP), dimerisation and translocation to the nucleus. The ER dimer binds to the estrogen response element (ERE) on the DNA and the coactivator (CoA) complex is formed. The ER is degraded at the proteasome (Prot) leading to altered gene transcription. P=phosphate and Ub=ubiquitin (from Zhou *et al.*, 2014 with permission).

1.3.2.2. The Non-classical Genomic Pathway

Alternative ER binding sites to the EREs have been identified. This involves direct interaction of the ERs with ER transcription factors where the ER does not bind promoter DNA (i.e. protein-protein interactions). This pathway is still induced via ligand activation of ER; however, complexes are formed with ligand induced activation of ER/specificity protein (Sp) and ER/activation protein 1 (AP-1). AP-1 is a complex of two oncogenic transcription factors that form a heterodimer, jun and Fos (Fig. 1.16) (Bjornstrom, *et al.*, 2005, Cheung, *et al.*, 2005). This indirect ERE interaction by the ER is the only difference between the classical and non-classical genomic signalling pathways.

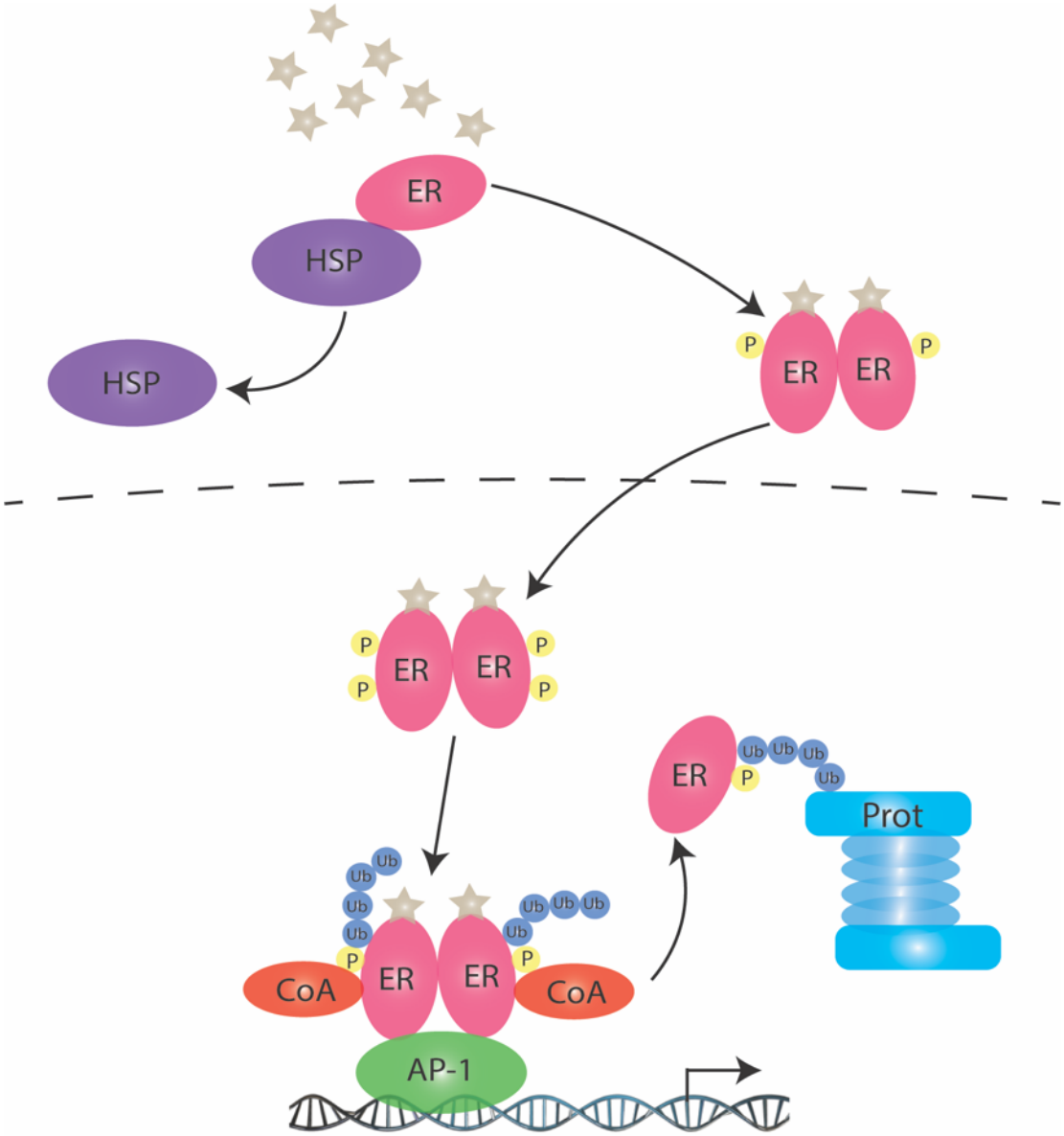


Figure 1.16: Illustration of the non-classical genomic signalling pathway. The ER monomer is activated upon E2 ( ) binding, facilitating dissociation from the heat shock protein (HSP), dimerisation and translocation to the nucleus. The ER dimer binds to the estrogen response element (ERE) on the DNA via an intermediate protein (AP-1) and the coactivator (CoA) complex is formed. The ER is degraded at the proteasome (Prot) leading to altered gene transcription. P=phosphate and Ub=ubiquitin (from Zhou *et al.*, 2014 with permission).

1.3.2.3. The Non-genomic Pathway

Several reports have shown the ability of ERs to interact with cytoplasmic kinases via crosstalk between cytoplasmic kinase pathways and genomic ER action. Various signalling pathways are activated upon E2 binding to ERs. These rapid events may be classified into four main signalling cascades: (i) phospholipase C (PLC)/protein kinase C (PKCs) (Ferret, *et al.*, 2001, Incerpi, *et al.*, 2003, Marino, *et al.*, 2001, Marino, *et al.*, 1998, Morley, *et al.*, 1992, Picotto, *et al.*, 1999), (ii) Ras/Raf/mitogen-activated protein kinase (MAPK) (Dos Santos, *et al.*, 2002, Klinge, *et al.*, 2005, Marino, *et al.*, 2002, Migliaccio, *et al.*, 2002, Russell, *et al.*, 2000, Tanaka, *et al.*, 2003, Watters, *et al.*, 1997, Woo, *et al.*, 2005), (iii) phosphatidyl inositol 3 kinase (PI3K)/AKT (Acconcia, *et al.*, 2005, Alexaki, *et al.*, 2006, Bjornstrom, *et al.*, 2005, Castoria, *et al.*, 1999, Castoria, *et al.*, 2001, Chambliss, *et al.*, 2005, Levin, 2005, Marino, *et al.*, 2005, Marino, *et al.*, 2003, Simoncini, *et al.*, 2000) and (iv) cAMP/protein kinase A (PKA) (Chen, *et al.*, 1998, Farhat, *et al.*, 1996, Gu, *et al.*, 1996, Malyala, *et al.*, 2005, Picotto, *et al.*, 1996, Picotto, *et al.*, 1999). These pathways present numerous interactions with several other pathways. For example, the ERα-E2 complex interactions with the IGF-1 receptor (IGF-1R), leading to IGF-1R activation and hence to MAPK signalling pathway activation (Kahlert, *et al.*, 2000). In addition, the ERα-E2 complex activates the epidermal growth factor receptor (EGFR) leading to an increase in the extracellular regulated kinases (ERK) and PI3K/AKT activities (Dos Santos, *et al.*, 2002, Driggers, *et al.*, 2002, Improta-Brears, *et al.*, 1999, Kupzig, *et al.*, 2005, Razandi, *et al.*, 2003, Zhang, *et al.*, 2004). These pathways also have a crucial role in the E2 action as a survival agent. They enhance the expression of bcl-2, an anti-apoptotic agent, block the activation of p38/MAPK, reducing pro-apoptotic caspase-3 activation and promote G1 to S phase transition via the enhancement of cyclin D1 expression (Fig. 1.17) (Acconcia, *et al.*, 2005, Marino, *et al.*, 2002, Marino, *et al.*, 2003).

ERβ has the opposite effect on these signalling pathways. It regulates E2 signalling when co-expressed with ERα, causing a concentration dependent reduction in ERα mediated transcription (Matthews, *et al.*, 2003). It also represses cyclin D1 expression and block ERα mediated induction when both ERs isoforms are present (Kilker, *et al.*, 2004, Liu, *et al.*, 2002). Recently ERβ has also been reported to induce persistent membrane-initiated activation of p38/MAPK without any interference on survival

proliferative pathways, thus restoring the balance with apoptosis (Acconcia*, et al.*, 2005). Human epidermal growth factor receptor 2 (HER2) can also be down regulated by ERβ and PTEN is upregulated, both of which lead to the down regulation of the PI3K/AKT pathway (Fig. 1.17) (Lindberg*, et al.*, 2011). ERβ has been considerably understudied in ER cell signalling and information is scarce. However, the significance of ERα signalling regulation is likely to have a major impact on these pathways.

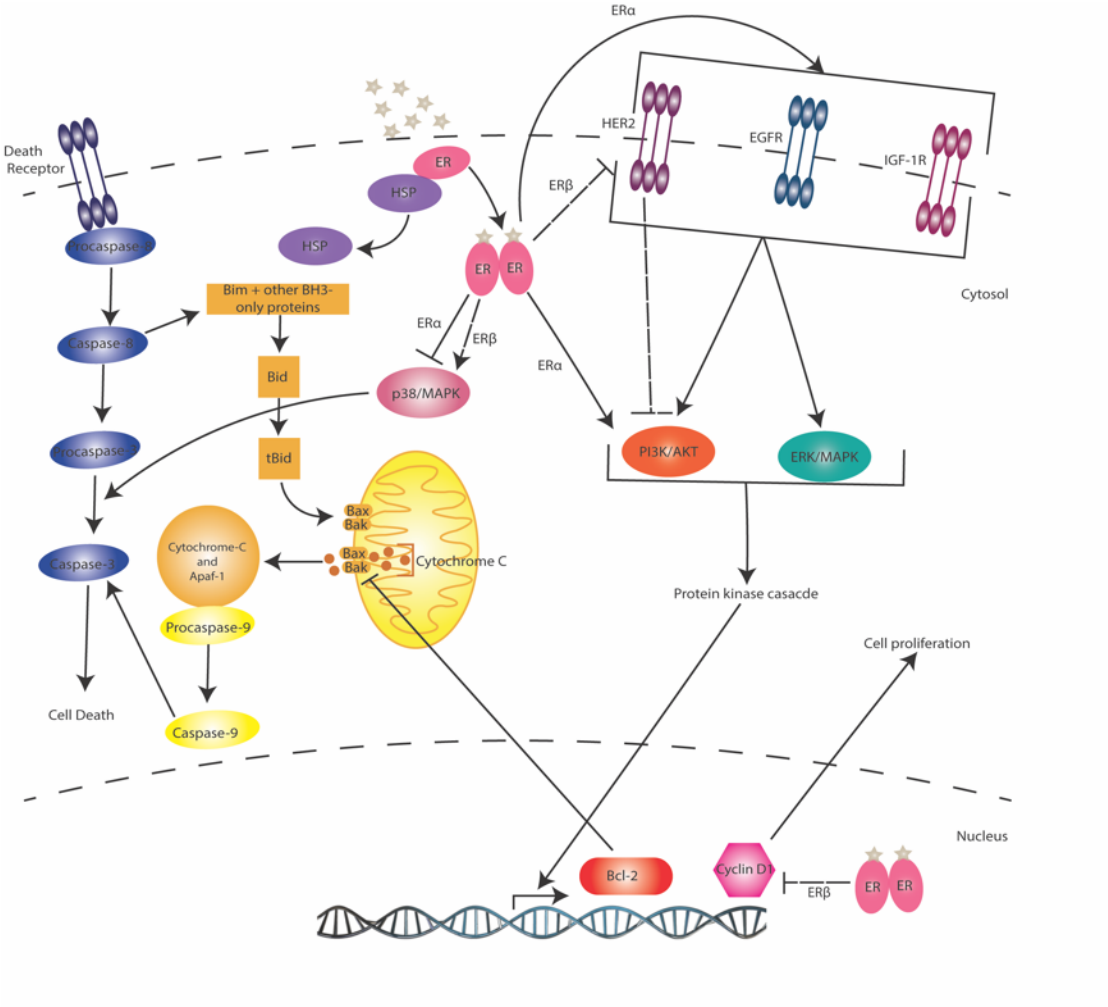


Figure 1.17: Illustration of the non-genomic signalling pathway. The ER monomer is activated upon E2 (✶) binding, facilitating dissociation from the heat shock protein (HSP) and dimerisation. The ER interacts with cell signalling pathways up/down-regulating processes such as apoptosis and cell proliferation (from Spencer, 2016 and Zhou *et al.*, 2014 with permission).

Clearly, ER actions are complex and importantly allow for tight regulation of estrogens in the body. However, the genomic and non-genomic pathways, although often studied independently, appear to be inherently linked. Therefore, when considering the actions of ERs in the non-genomic pathway (e.g. cell signalling pathways), not only does the cell signalling pathway become activated but in turn the ER is post-translationally modified (e.g. phosphorylated) which promotes the genomic pathway. For example, ligand bound ERα binds to SRC and Pi3K complexes leading to AKT and MAPK activation. In turn, ERα is post translationally modified at sites such as Try537 which allows recruitment of the E3 ligase, E6 associated protein (E6AP), and initiates ER proteolysis and transcriptional activation (Fig.1.18).

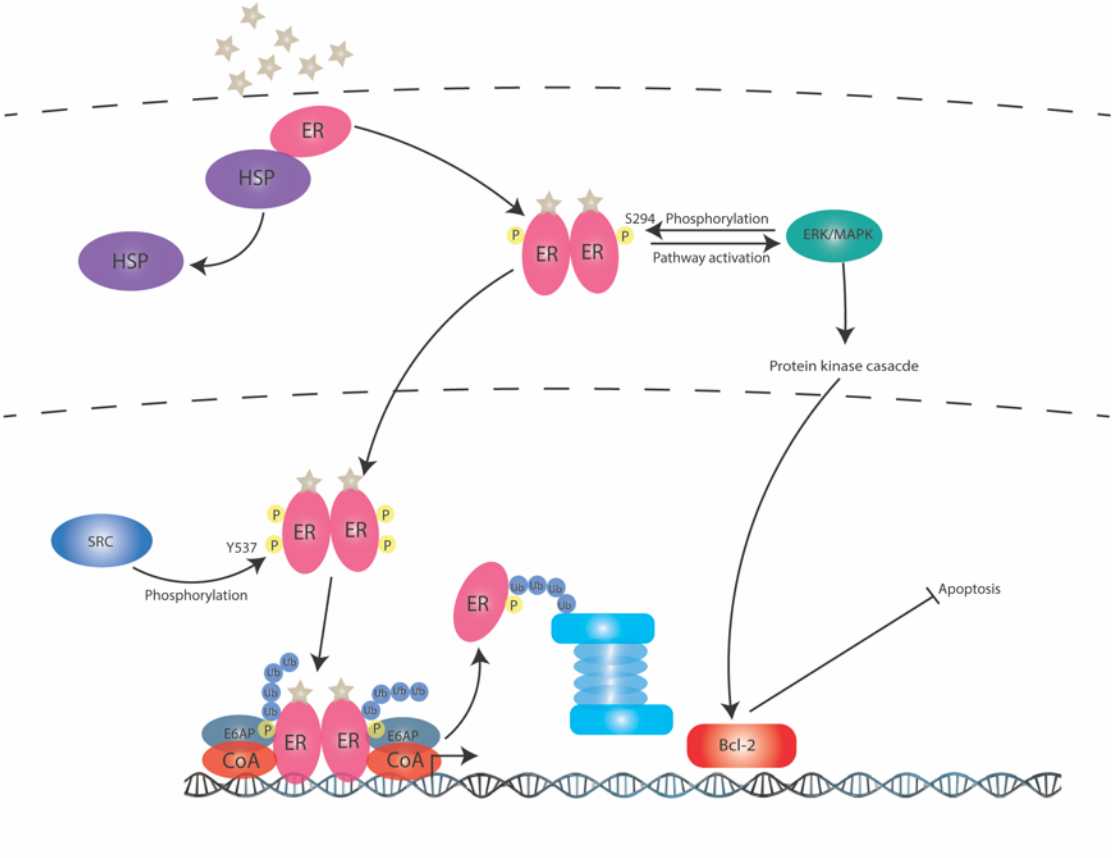


Figure 1.18: Illustration of the interplay between genomic and non-genomic signalling pathways. The ER dimer is phosphorylated by kinase proteins, which is required for genomic signalling, whilst activating non-genomic signalling pathways. E2 = , CoA= coactivator complex, Prot= proteasome, P=phosphate and Ub=ubiquitin. (Elements of this diagram were derived from Zhou *et al*., 2014. The concept of the entire diagram is the author's).

This is just one example of the many post translational modifications of the ER which influence the stability, subcellular localisation, transcriptional activity and hormone sensitivity of the ligand activated ER-transcriptional apparatus. There are approximately 29 sites on the ER that undergo either phosphorylation, methylation, acetylation, sumoylation, palmitoylation or ubiquitylation (Le Romancer*, et al.*, 2011). Activation of these sites drives rapid signalling kinase cascades which lead to non-genomic mitogenic effects. These post translational events modulate ER function by altering its binding to ligands, to target gene promoters or to ER coactivators (Campbell*, et al.*, 2001, Legoff*, et al.*, 1994, Likhite*, et al.*, 2006, Rayala*, et al.*, 2006).

Altering these key ER functions can influence gene expression and the genomic pathway. There are many other interactions between hormonal and growth factor signalling pathways. These multiple signalling pathways downstream of receptor tyrosine kinases (e.g. EGRF) and insulin-like growth factor 1 receptor (IGF1R)) co-ordinately regulate the dynamics of the ER mediated transcriptional regulation. MAPK mediates ER phosphorylation at S294, and cyclin E-cyclin dependent kinase 2 (CDK2) phosphorylates ER at S342 to prime the ER-S-phase kinase associated protein 2 (SKP2) interaction.

One of the key roles for post translational modifications of the ER is aiding in the formation of the coactivator complex and recruitment of proteins to the complex. There is a series of post translational modifications that lead to the recruitment of the transcriptional machinery and the degradation of the ER to the proteasome. The coactivators often have enzymatic activities which enable them to acetylate, methylate or demethylate (Lonard*, et al.*, 2007), helping overcome the physical constraints of the highly coordinated chromatin encased template (Zhou*, et al.*, 2014). Coactivation allows recruitment of the transcriptional machinery and thus gene transcription. The coactivators then recruit the appropriate ubiquitination proteins (E1, E2 and E3 ligases) which makes a polyubiquitin chain and thus signals to the proteasome that the ER is ready for degradation.

These actions are not isolated to endogenous estrogens and antagonists. They can be induced by compounds that mimic E2, known as xenoestrogens.

1.4. Xenoestrogens

Xenoestrogens (from the Greek ξένος 'xenos' meaning foreign) are compounds that structurally mimic E2. To date, over 160 xenoestrogens have been identified (Brody, *et al.*, 2006, Brody, *et al.*, 2003, Brody, *et al.*, 2007), being either natural or synthetic, many found in the environment (e.g. food). The chemical attributes for E2 mimicry are an aliphatic hydroxyl group (corresponding to the 17β-hydroxyl of E2) and an aromatic hydroxyl group (corresponding to the A ring 3-hydroxyl group of E2) separated by approximately 10 Å of hydrophobicity in the correct three-dimensional spatial arrangement (Ye, *et al.*, 2018). In general, xenoestrogens are considered ER agonists but each has a different binding affinity; so, the cellular response they trigger depends on their individual binding affinity – this is termed estrogenicity. There have been numerous studies on the adverse effects of xenoestrogens in both animal and human populations. In animals they have been associated with reduced penis length and low levels of testosterone in American Alligators (*Alligator mississippiensis)* (Guillette, *et al.*, 1996), imposex in molluscs (e.g. dog whelks (*Nucella lapillus)*) (Davies, *et al.*, 1997) and vitellogenin biosynthesis in male fish (Kidd, *et al.*, 2007, Sumpter, *et al.*, 1995).

In humans, xenoestrogens are thought to be one of the leading causes of adverse effects in human health and development due to their frequency in occurrence in the environment (UNEP/WHO, 2013). Studies have shown the profound effects of xenoestrogen exposure on the development and function of the male reproductive tract, namely abnormalities including hypospadias and cryptorchidism (Aksglaede, *et al.*, 2006). The most notable finding was the increased incidence of hypospadias in sons of women consuming a vegetarian diet during pregnancy (North, *et al.*, 2000). Indeed, vegetarians do not eat meat which makes soy and beans their main source of protein. Soy and beans are a major source of isoflavones (Kaufman, *et al.*, 1997); therefore, it is possible that vegetarians are exposed to more xenoestrogens. A number of studies have also linked the decline in the male sperm count to xenoestrogen exposure, suggesting a decline between 1.5% and 3% per year (Carlsen, *et al.*, 1992, Levine, *et al.*, 2017, Swan, *et al.*, 2000). The age of puberty has decreased over the last century, with the average age being 12 years old in the USA, down from 16-17 years old at the turn of the 20th century (Pierce, *et al.*, 2012). The onset of puberty is regulated by gonadotrophins which in turn stimulate an increase in estrogen levels

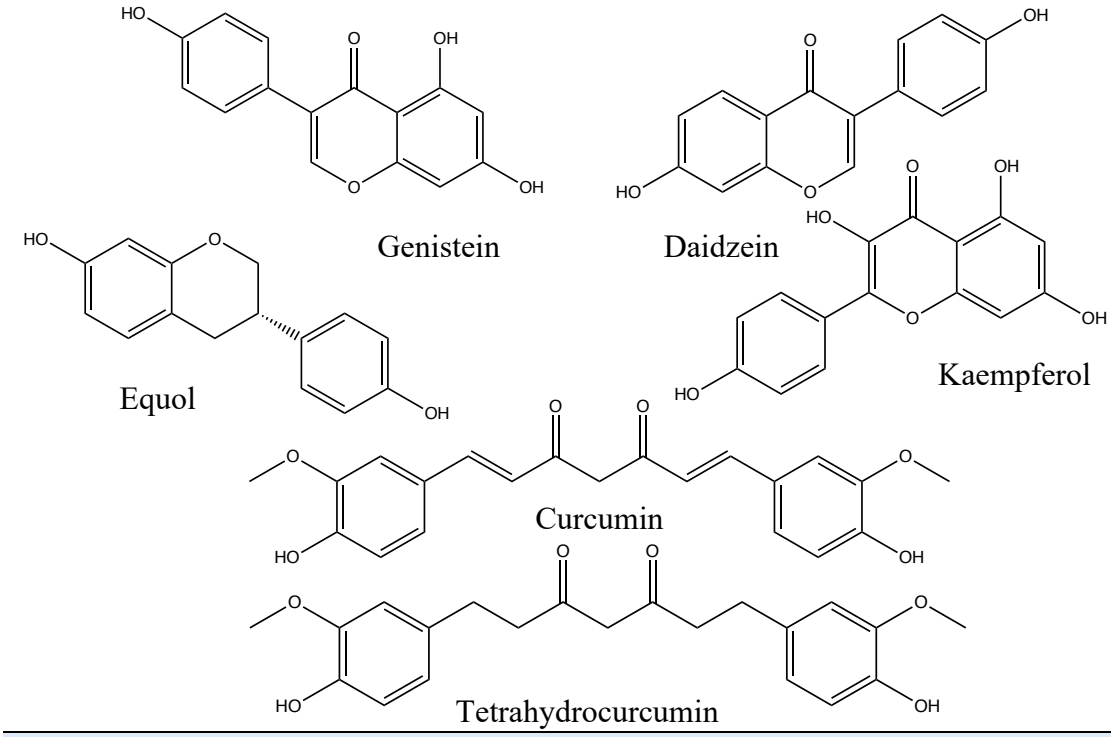
(MacGillivray, 2004). Unsurprisingly, xenoestrogens have been suggested as one of the main reasons for this change but of course, there have been changes in diet and nutrition during that time which could at least in part explain this (Willett, 2013).

Sex-linked cancers are one of the most studied adverse human health effects associated with xenoestrogen exposure. Breast, testicular and prostate tissues all express ERs and therefore, respond to xenoestrogens in the same way it would to estrogens – proliferation. It is not unexpected that xenoestrogens can cause proliferation and thus the initiation and progression of cancer cells (Fernandez*, et al.*, 2010, Forouzanfar*, et al.*, 2011).

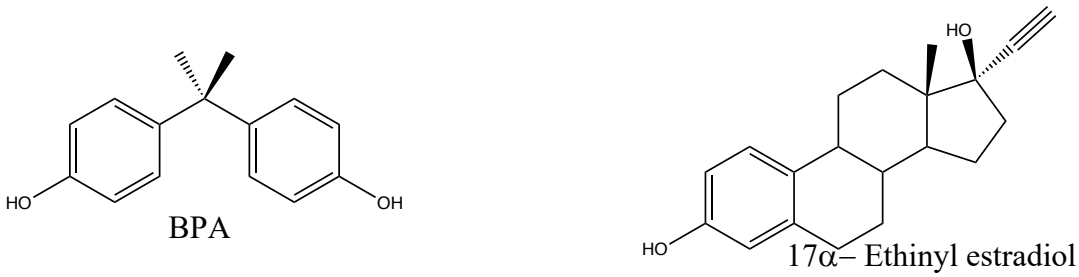
Some xenoestrogens (Fig. 1.19) are components of cosmetics, personal care products and pharmaceuticals, and are found naturally in food, as well as contaminants being in food and water, so exposure occurs by several routes including ingestion (Allmyr*, et al.*, 2006, Allmyr*, et al.*, 2008, Dayan, 2007, Muller*, et al.*, 1998, Waring*, et al.*, 2008), absorption through the skin (Chedgzoy*, et al.*, 2002, Darbre*, et al.*, 2008, Dayan, 2007, Hayden*, et al.*, 1997, Jungbauer*, et al.*, 2014) and inhalation (Rudel*, et al.*, 2009). Once xenoestrogens reach the bloodstream, they are subject to a variety of metabolic reactions designed to facilitate elimination from the body. It is also possible for metabolites to be estrogenic (Zalko*, et al.*, 2003). Currently, most chemicals that are regulated, are regulated individually and safe levels determined (e.g. NOAEL); however, exposures to environmental chemicals is usually part of complex cocktail involving potentially combined effects – these cocktails are not regulated. In the context of xenoestrogens, since the individual components of a cocktail work through the same receptor interaction, it is very likely they will at least be additive. Therefore, using individual exposure safe levels could be very misleading (Eubanks, 2004).

Xenoestrogen

Phytoestrogens



Plastics and Pharmaceuticals



Parabens

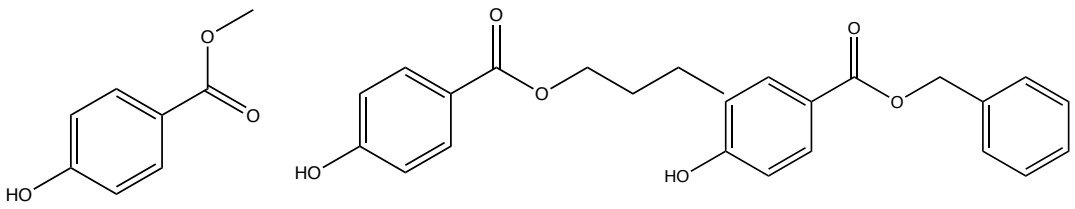


Figure 1.19: Molecular structures of selected xenoestrogens.

1.4.1. Phytoestrogens

Phytoestrogens (from the Greek φυτόν 'phytos' meaning plant) are plant derived xenoestrogens, naturally occurring in foods such as beans and other legumes, grains, green vegetables (e.g. Brussel sprouts and spinach) and coffee (Grace, *et al.*, 2003). They mainly belong to a large group of substituted natural phenolic compounds: the coumestans, prenylflavoniods and isoflavones. The most extensively researched are the isoflavones, commonly found in soy beans. Other phytoestrogens include the lignans and curcuminoids. Phytoestrogens were first observed in 1926 but have been extensively used in traditional medicines. Women have long used the hop plant (*Humulus lupulus*), which contains the prenylflavoniod 8-prenylnaringenin, to initiate their menstrual cycle (Verzele, 1986).

Phytoestrogens exert their primary effects through ER binding and have a high structural analogy to E2. Interestingly, many phytoestrogens preferentially bind to ERβ over ERα (Kuiper, *et al.*, 1997, Lehmann, *et al.*, 2008). It is thought that this preference is a result of the spatial arrangement of the hydroxyl groups.

In addition, phytoestrogens have been associated with protective actions against a variety of cancers, cardiovascular disease and osteoporosis (Adlercreutz, 2002, Bhathena, *et al.*, 2002, Cederroth, *et al.*, 2009, Hughes, 1988, Jungbauer, *et al.*, 2014, Karahalil, 2005, Patisaul, *et al.*, 2010, Zhao, *et al.*, 2011). The health benefits and potential adverse effects attributed to phytoestrogens are varied and controversial (Andres, *et al.*, 2011, Bennetau-Pelissero, 2016, Rietjens, *et al.*, 2017, Rietjens, *et al.*, 2013, Wuttke, *et al.*, 2006). This has contributed to the lack of regulations around xenoestrogens in general, especially in mixtures.

Genistein

Genistein is a major isoflavone found in beans, including soy beans, and some green vegetables (e.g. spinach) (Coward, *et al.*, 1993). Soy flour is commonly used in food manufacturing and is widely used in breads and cereals (Farzana, *et al.*, 2015). Genistein preferentially binds to ERβ over ERα (Kuiper, *et al.*, 1997). Genistein has been extensively studied and is known for its possible protective effects against breast and prostate cancer (Spagnuolo, *et al.*, 2015). However, there is a concentration dependence to the protective effects, where genistein is only protective at high exposure concentrations and at low exposure concentrations it promotes cancer cell

growth. Studies in MCF-7 cells show that exposure concentrations in the nanomolar range promoted cell proliferation, while concentrations in the micromolar range had an anti-proliferative effect. Interestingly, when E2 is present nanomolar concentrations of genistein induce an anti-proliferative effect (Chang, *et al.*, 2008). Similar outcomes have been observed in women of child bearing age (e.g. high E2 levels) experiencing protective effects against breast cancer when exposed to genistein compared to post-menopausal women (e.g. low E2 levels) who experience promotive effects when exposed to genistein (Rice, *et al.*, 2006).

Daidzein

Daidzein is also a major isoflavone found in soy and soy based products (Coward, *et al.*, 1993). Daidzein also preferentially binds to ERβ and has similar protective effects to genistein (Kostelac, *et al.*, 2003). Daidzein and genistein are very similar structurally and only differ by the extra hydroxyl group at carbon atom 5 on genistein (Fig. 1.20). Therefore, it is not surprising that they elicit similar protective effects.

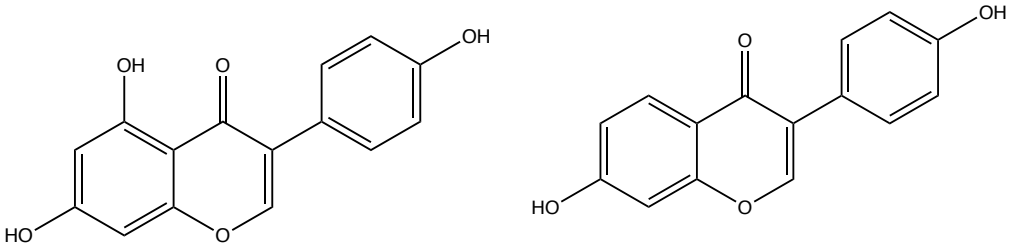


Figure 1.20: Structures of genistein (left) and daidzein (right).

Equol

Equol is a metabolite of daidzein produced by bacteria present in the human gut (Moors, *et al.*, 2007). Two isomers of equol exist, but the gut bacteria only produce the S-isomer which is estrogenic (Fig. 1.21). It is more estrogenic than daidzein (Sathyamoorthy, *et al.*, 1997). Interestingly, only about 30-50% of people have the intestinal bacteria that make equol, so equol is only found in a certain percentage of the human population (Sanchez-Calvo, *et al.*, 2013). The S-isomer of equol preferentially binds to ERβ, as with its parent compound daidzein (Muthyala, *et al.*, 2004).

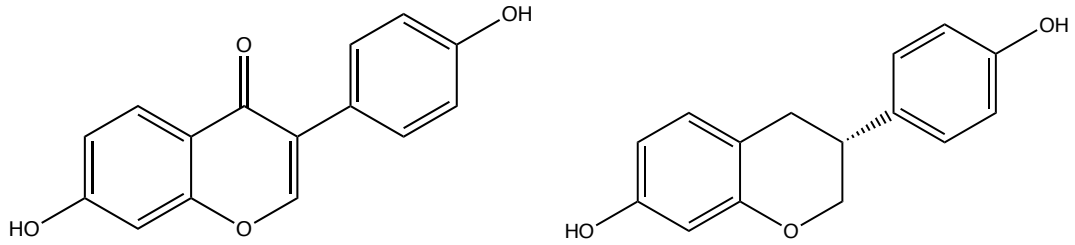


Figure 1.21: Daidzein is metabolised to S-equol by human gut bacteria

Kaempferol

Kaempferol is a flavonoid found in fruit and vegetables such as broccoli, apples, strawberries and beans (Somerset*, et al.*, 2008). Kaempferol has not received a lot of attention until recently when studies have suggested it has anti-cancer properties. Kaempferol has been demonstrated to invoke the downregulation of various cell signalling pathways that inhibit cancer development (Ramos, 2007). Studies in MCF-7 cells have shown that kaempferol has both estrogenic and anti-estrogenic activity. As for the observed responses with other phytoestrogens, kaempferol induces MCF-7 cell proliferation at low concentrations, with anti-proliferative effects at high concentrations or in combination with E2 (Oh*, et al.*, 2006). Therefore, it has also been earmarked as a potential breast cancer therapeutic. Unsurprisingly, kaempferol also has a preference for ERβ over ERα (Kuiper*, et al.*, 1998).

Curcumin

Curcumin is the principal curcuminoid of turmeric and is often sold as a herbal supplement. It is also used in cosmetics, food flavouring, and food colouring (Aggarwal*, et al.*, 2003). Curcumin is a tautomeric compound existing in enolic form in organic solvents and as the keto form in water (Fig.1.22) (Bertolasi*, et al.*, 2008, Nie*, et al.*, 2008). Curcumin has been extensively studied in laboratory and clinical studies and is thought to be a potential cancer therapeutic (Adiwidjaja*, et al.*, 2017). Again, like other phytoestrogens, it has been shown to have concentration-dependent proliferative/anti-proliferative effects in MCF-7 cell studies (Lv*, et al.*, 2014). However, the bioavailability is low and therefore, curcumin has not progressed into therapeutic clinical studies to date (Hatcher*, et al.*, 2008, Ireson*, et al.*, 2001, Ireson*, et al.*, 2002).

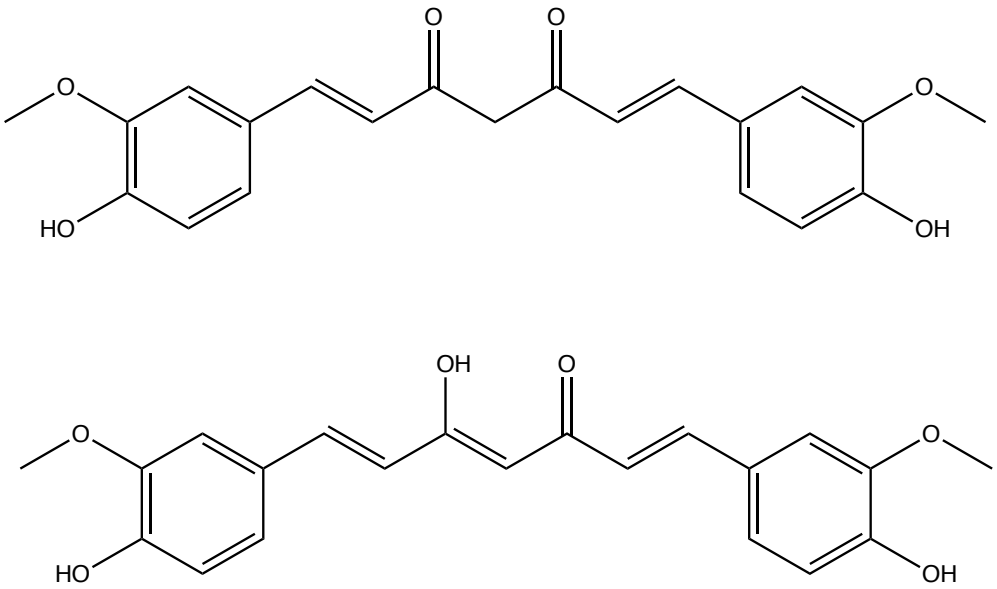


Figure 1.22: Structures of the curcumin enolic and keto stereoisomers.

Tetrahydrocurcumin

Tetrahydrocurcumin is a metabolite of curcumin, but is much more stable, and easily absorbed by the gastrointestinal tract due to the lack of double bonds beside the keto groups (Okada*, et al.*, 2001). It is thought that tetrahydrocurcumin is one of the genuinely bioavailable forms of curcumin, and therefore, the compound responsible for the anticancer properties observed in some curcumin studies (Han*, et al.*, 2016). However, its potential anticancer properties have only recently been observed, and are not well understood. Tetrahydrocurcumin is known to have dose-dependent anti-proliferative effects in MCF-7 cells (Han*, et al.*, 2016). Although these anti-proliferative effects have been suggested to be a result of the activation of apoptosis, it is clear that this could be a result of non-genomic ER actions as described in section 1.3.2.3.

1.4.2. Synthetic Xenoestrogens

Synthetic xenoestrogens are widely used in industrial compounds, such as BPA, and pharmaceuticals, such as EE2. Synthetic xenoestrogens elicit their effects via ERs which lead to down-stream biological effects such as cell proliferation. Interestingly, the public often perceive synthetic xenoestrogens to be more risky than natural xenoestrogens, even though they act via the same mechanism.

Bisphenol A

Bisphenol A (BPA) is a monomer used to manufacture polycarbonate plastics (Fig. 1.2 and 1.23) and epoxy resins used in protective coatings of food cans and sealants in dentistry (Calafat*, et al.*, 2005). BPA is the xenoestrogen that has received the most public attention, leading to WHO enquiries into its safety (EFSA, 2015, WHO, 2010). Although it is deemed safe due to its high rate of metabolism, many countries including Canada, USA, China, and Brazil have made an unprecedented move to ban BPA from being used to manufacture polycarbonate plastic babies' bottles on the grounds that milk formula-fed babies are likely to be a key target group due to their sensitive developmental stage, high BPA intake per body weight, and their sole source of food likely contaminated with BPA (Almeida*, et al.*, 2018, JRC/IHCP, 2010). Unlike most xenoestrogens, BPA does not have an ER isoform preference, and binds ERα and ERβ with similar affinity. BPA is 1000-2000 fold less potent than E2 itself (Bonefeld-Jorgensen*, et al.*, 2001).

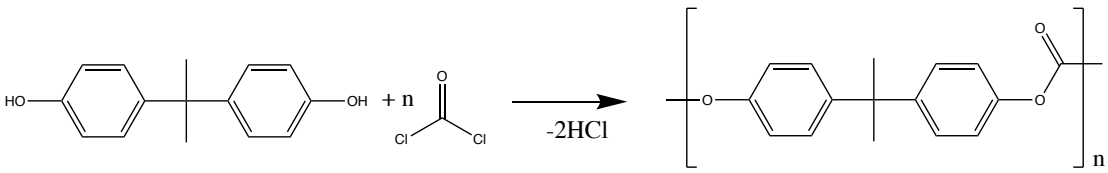


Figure 1.23: BPA and phosgene react to give a polycarbonate under biphasic conditions.

EE2

Ethinylestradiol (EE2) is the synthetic estrogen used in the oral contraceptive (OC) or "The Pill". The primary role of the OC is to control ovulation and in turn prevent implantation and pregnancy (Tata, 2005). It is designed to be a potent estrogen, with its potency owed to its slow metabolism due to the triple carbon-carbon bond and high

structural analogy with E2 (Levin, 2005). EE2 preferentially binds to ERα and is 4 times more potent than E2. EE2, although well known for its use in preventing pregnancy, is also known for its effects on aquatic environments. It is not well degraded in sewage treatment plants and thus gets released into the aquatic environments where it comes into contact with fish and aquatic species. It has been shown that male fish living in such areas synthesise vitellogenin, a protein normally synthesised by females during the development of the ovum, and bizarrely, early stage egg production in the testes (Kidd*, et al.*, 2007, Sumpter*, et al.*, 1995). Although second hand human exposure is minimal, the effluents do have a tendency to leach into drinking water supplies. A well-known example is the River Thames in London, England. The River Thames is the main water source for the city, however, it also has 352 sewage treatment plants treating sewage from around 5.4 million people, discharging into the river every year (Williams*, et al.*, 2009). Therefore, the bioaccumulation of EE2 could mean populations are being exposed to increasing concentrations of EE2 in their drinking water, although these concentrations are still in the nanomolar order of magnitude.

Parabens

Parabens (esters of 4-hydroxybenzoic acid) are used as preservatives in cosmetics, topical pharmaceuticals and personal care products such as moisturisers and shampoos, and in foods such as jams and baked goods (Rudel*, et al.*, 2009). They prevent bacterial and fungal growth in these products (Darbre*, et al.*, 2008). Parabens are widely used due to their low toxicity and low estrogenicity (Anderson, 1994, Anderson, 1995, Soni*, et al.*, 2001, Soni*, et al.*, 2002). The longer chained parabens are more estrogenic than their shorter chained counterparts, owing this increased estrogenicity to better occupancy of the ERs (Karpuzoglu*, et al.*, 2013).

Methylparaben

Methylparaben is the methylester of hydroxybenzoic acid. It is widely used in a variety of cosmetics and personal care products. It is one of the most commonly used parabens due to its seemingly non-existent estrogenicity. Therefore, it is considered safe by The Food and Drug Administration (FDA) for food and cosmetic antibacterial preservation (Soni*, et al.*, 2002).

Butylparaben

Butylparaben is butylester of 4-hydroxybenzoic acid. It is a highly successful antimicrobial preservative in cosmetics. It is also used in medication suspensions and flavouring additives in foods. Butylparaben has displayed estrogenicity in most competitive binding assays and is more estrogenic compared to the short chain parabens (methyl, ethyl and propyl) (Terasaki*, et al.*, 2009).

Benzylparaben

Benzylparaben is the benzylester of hydroxybenzoic acid and is one of the more recent parabens used in cosmetics as an antimicrobial preservative. Benzylparaben has been reported as having estrogenic potential but no anti-estrogenic potential in a panel of ER mediated assays, including MCF-7 cells (Darbre*, et al.*, 2003). It is not surprising that benzylparaben has estrogenic properties given that the possible cytochrome P450 generated metabolite of a hydroxyl group added to the benzyl ring (Fig. 1.24). If this occurred this would make benzylparaben the most similar to E2 of all the parabens.

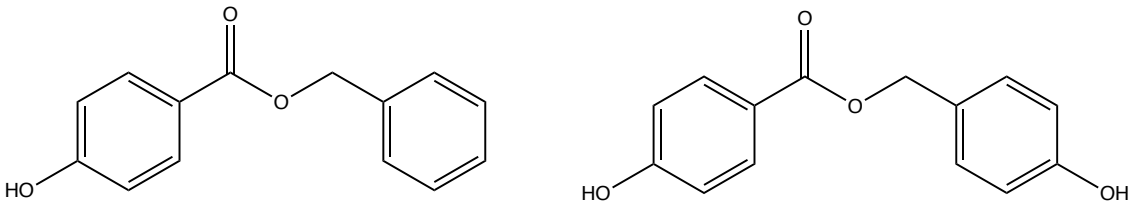


Figure 1.24: Structures of benzylparaben and its potential metabolite.

1.4.4. Exposure to Xenoestrogens

Clearly, xenoestrogens are found in a lot of common products, to which humans are exposed in their day-to-day lives. Graham (2012) compiled an extensive literature search which demonstrated the levels of xenoestrogens found in the urine of adults (Fig. 1.25). Urine was chosen as it is the most common method of determining xenoestrogen exposure in adults. The total concentration (free + conjugated) is most often reported. These data represent cohorts of different sizes and nationalities, and thus a great diversity in lifestyles. The data shown in Fig. 1.25 includes almost all of the xenoestrogens of interest for this study.

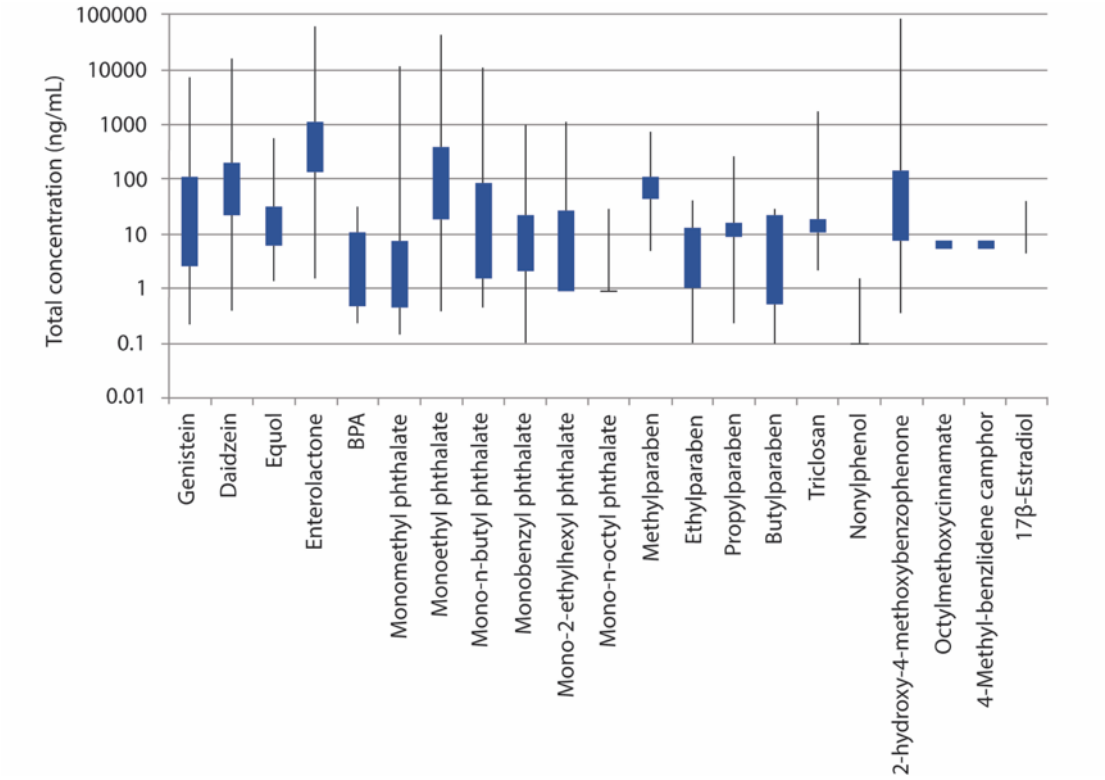


Figure 1.25: Range of reported total xenoestrogen concentrations in adult human urine. Lines indicate range of reported values, bars indicate lowest and highest median reported values (from Graham 2012 with permission).

1.4.5. Exposure to Xenoestrogen Cocktails

It is clear from the information above that co-exposure to xenoestrogens and possible bioaccumulation are realistic scenarios, because humans are constantly exposed to various xenoestrogens simultaneously through the environment and diet. Therefore, in linking negative health effects to xenoestrogen exposure, it may be more relevant to consider mixtures rather than single substances. Exposure to xenoestrogens is an exposure mixture of changing composition and concentration. Because the endpoint of concern is hormonal signalling, there is no safe threshold below which there is no activity (Brucker-Davis*, et al.*, 2001). When assuming a similar mode of action for mixture components, researchers have concluded that these mixture effects are often described most accurately by the concept of the concentration addition model (CA) postulated by Loewe and Muischnek in 1926, making it the most commonly used model in xenoestrogen mixture research. This simplest approach to assessing the total estrogenic effect of mixtures is where each component adds to the total proportion of the concentration weighted by its relative potency. Rajapakse et al demonstrated in

principle that every xenoestrogen, however weak, may add incrementally to the total estrogenic effect, even at very low concentrations, and even in the presence of potent endogenous steroidal estrogens (Rajapakse*, et al.*, 2002).

However, there are inherent mathematical limitations to the concept of CA regarding the prediction of effects caused by mixtures of substances differing in efficacy (maximal response). This limitation to the maximal effect level of the mixture component with the lowest efficacy which leads to predictive blind zones (Liu*, et al.*, 2015). Furthermore, if mixture components have no effect, the predictivity of CA is disturbed. Currently, the accepted CA model (discussed further in Chapter 5) relies upon the theory that since estrogenic compounds are likely to be working through the same mechanism (i.e. binding to the ER) they have got to be at least additive. However, this is perhaps flawed because it does not consider their binding constants. This could modify the additive effect.

1.4.6. Measurement of Xenoestrogens

Many *in vitro* assays measure endocrine activity at the molecular level e.g. nuclear receptor activation. Whether such activation indeed leads to biologically relevant adverse outcomes depends on key events in the respective adverse outcome pathways. Adverse health effects, such as compromised reproduction and carcinogenesis, have been attributed to the disruption of the E2 signalling pathway (Diamanti-Kandarakis*, et al.*, 2009). ER activation and E2-induced proliferation of ER positive cells are considered hallmarks of estrogenic activity. Well established on the one hand are ER mediated reporter gene assays that measure ER binding and transactivation by the substance of interest, thus estimating its impact on the genomic ER pathway. On the other hand, the E-SCREEN assay, based on the ER-expressing breast cancer cell line MCF-7, responds with increased proliferation to estrogenic compounds and thus is commonly used to estimate the proliferative potential of xenoestrogens. This response can be mediated by both genomic and non-genomic ER pathways. Interestingly, both assays have been frequently used independently, but few studies consider both together (Evans*, et al.*, 2012).

It remains to be clarified if findings derived from ER-dependent reporter gene assays are sufficient to predict effects at higher levels of cellular organisation. It is also possible for mixtures to have a higher potency than that predicted by the simple

additivity model of CA. This behaviour is described as synergy. For example, mixtures of benzophenones (xenoestrogens found in sunscreens and lotions), alone or including E2, showed synergy, with substantial increases in observed effect in the YES assay even though each UV filter was present at its no observable effect concentration (NOEC) level (Kunz*, et al.*, 2006). In addition, mixtures with phytoestrogens, particularly genistein, have been well documented to induce a supramaximal effect (i.e. an effect greater that are greater than 100%) in luciferase-based reporter gene assays, which can be confused with synergism (Sotoca*, et al.*, 2010). However, although it appears synergistic, it is unlikely that genistein would induce that level of response, especially in comparison to E2. Therefore, the effect is likely an artefact of the assay (Montano*, et al.*, 2010). This poses a concern when using gene reporter assays to regulate xenoestrogen mixtures and thus care should be taken when using data from these experiments.

1.4.7. Assessing the risk of Xenoestrogen cocktails

The complexity of dealing with mixtures in exposure assessment is recognised, but appropriate methodologies for the assessment risk due to exposure mixtures are still hotly debated (Filby*, et al.*, 2007, Kortenkamp, 2007, Kortenkamp, 2008, Kortenkamp*, et al.*, 2007, Payne*, et al.*, 2000, Rajapakse*, et al.*, 2002). Nevertheless, some regulatory frameworks are beginning to consider groups of chemicals that act via the same mechanism collectively rather than evaluating the potential risks individually.

One approach to simplifying the topic of endocrine disruption is to treat the effects of exposure individually according to the signalling pathway, e.g. E2 signalling disruption separate from androgen or thyroid disruption. While this approach disregards the potential for the different signalling pathways to interact, e.g. anti-androgenic effects contributing to an overall estrogenic outcome, there is insufficient information available at this time to completely address the problem. An example of this is the parabens. They simultaneously upregulate ER gene expression, downregulate AR gene expression and inhibit aromatase activity, thereby supressing the biosynthesis of estrogens. Parabens can contribute to the common effect (feminisation) via two distinct mechanisms (estrogenicity and anti-androgenicity) and also contribute to a contradicting effect (masculinisation) via supressing endogenous estrogen biosynthesis (Engeli*, et al.*, 2017, Vo*, et al.*, 2009). Similar behaviour is

likely present with other classes of compounds of interest to estrogenic signalling disruption, but the data are currently lacking.

This study will be limited to assessing xenoestrogen mixtures in the context of estrogen signalling using a variety of the ER model systems discussed above. This will be done by using the CA model, gene reporter assays that examine both ERα and ERβ individually, and the breast cancer cell line, MCF-7 to encompass the effects individually by ERs and in the context of breast cancer.

1.4.8. Xenoestrogens as Cancer Risk Factors

Arguably, one of the deadliest linkages between xenoestrogens and human health is cancer. While there are some well-known associations (e.g. breast cancer) (Jenkins*, et al.*, 2012), there is mounting evidence that suggests a wider range of cancers could be influenced by xenoestrogen exposure, thus xenoestrogens could be risk factors for multiple cancers (Fucic*, et al.*, 2012). Lung cancer has long been linked to smoking, however, with the number of smokers decreasing a clear gender bias is emerging. The ratio between ERα and ERβ in the lung tissue seems to be relevant for lung cancer development and might explain the higher incidence of lung adenocarcinoma in women compared to men (Whitrow*, et al.*, 2003). Currently, ERβ positive lung cancer in men is associated with a significantly lower mortality rate compared to ERβ negative lung cancer (Schwartz*, et al.*, 2005). Interestingly, women taking HRT have shown an increased lung cancer incidence, highlighting a correlation between lung cancer and estrogens (Slatore*, et al.*, 2010). Of course, it is possible that mutations in other genes (e.g. EGRF) could lead to increased expression and therefore, increased proliferation (da Cunha Santos*, et al.*, 2011). Xenoestrogens may also play a role in lung cancer incidence and could be a risk factor.

There is also ample evidence that suggests estrogens may play a critical role in predisposing or even causing prostate cancer. However, studies have shown mixed results (Nelles*, et al.*, 2011) with one study finding an association between elevated plasma estrogens and an increased risk of prostate cancer, and another study finding the opposite where a decrease in estradiol levels was associated with prostate cancer. It is not hard to envision the influence of estrogens/xenoestrogens on the prostate gland, particularly since the prostate expresses ERβ.

Similar mechanistic correlations have been made for kidney (Kabaria, *et al.*, 2016, Tanaka, *et al.*, 2003), pancreatic (Konduri, *et al.*, 2007, Lowenfels, *et al.*, 2006, Sauerland, *et al.*, 2009), colon (Caiazza, *et al.*, 2007, Hartman, *et al.*, 2010, Rudolph, *et al.*, 2012) and brain (Batistatou, *et al.*, 2006) cancer. Therefore, although the primary focus of this thesis is on breast cancer, it is important to consider the observed effects in the context of many other cancers.

1.5 Breast Cancer

Cancer originating in the mammary gland is the most common type of cancer in women. The lifetime risk for a woman in developed countries has been calculated at around 1 in 7 to 1 in 10. When it comes to New Zealand, the latest analysis reports an accumulated lifetime risk of developing breast cancer to be 1 in 9 with approximately 600 women dying from the disease every year (MOH, 2015). This means around 11% of the New Zealand female population will be diagnosed with breast cancer at some point in their life (calculated from the average lifespan 0-74 years; e.g. the risk of developing breast cancer before the age of 74 years).

1.5.1. Breast Cancer Biology

At normal physiological conditions, the epithelium is a well-defined structure where epithelial cells form the lining of ducts that are responsible for milk transport during lactation (Fig. 1.26). Ductal carcinoma is the most common type of breast cancer, occurring both in women and men, although the incidence in men is low. Contrasting with healthy tissues, cells within solid tumours acquire several pathophysiological characteristics that confer them survival, proliferative and migratory capabilities. Tumour progression is characterised by a mass formed by multiple populations of cells with mechanisms capable of inhabiting apoptosis, while promoting survival pathways and the invasion of healthy tissues through the blood and lymphatic system. Morphologic and functional alterations of epithelial cells, as well as alterations of important surface molecule expression, are crucial for tumour initiation and development.

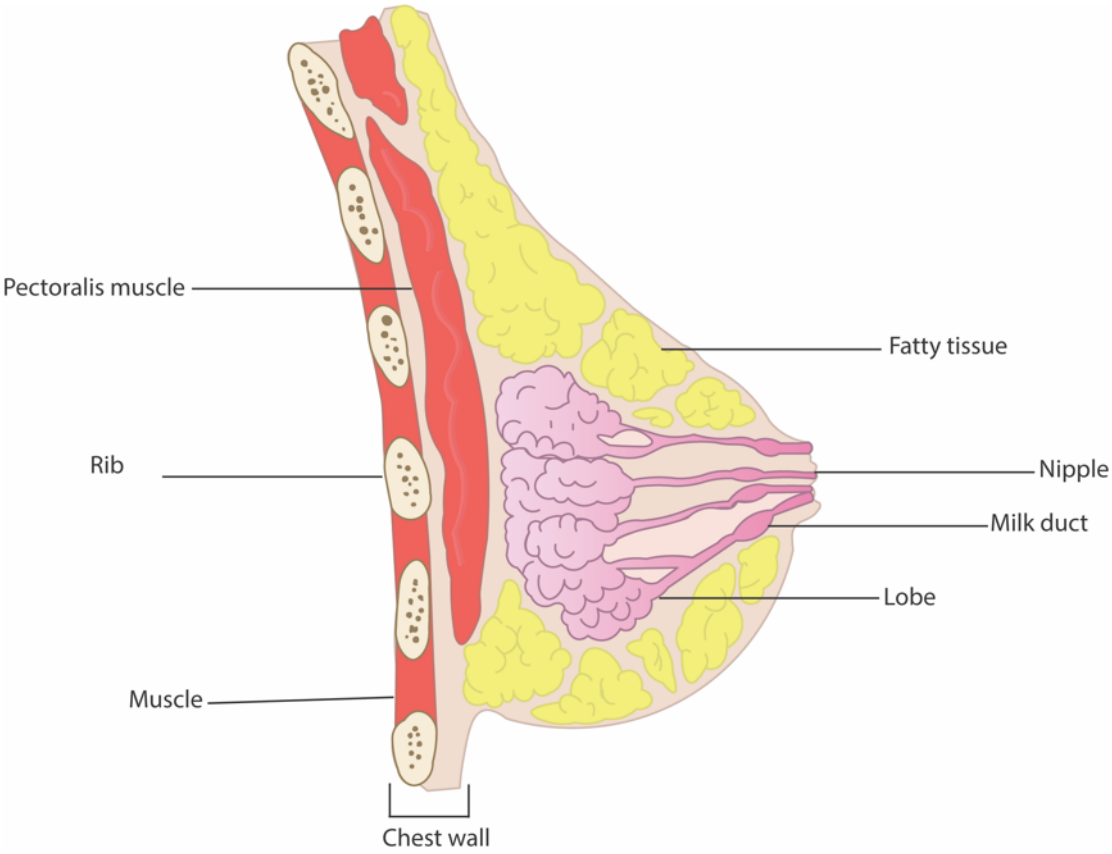


Figure 1.26: Breast Parenchyma (from https://www.britannica.com)

When normal epithelium has begun malignant transformation, it undergoes a first stage of excessive proliferation known as hyperplasia followed by the appearance of cells showing aberrant characteristics (atypical ductal or lobular hyperplasia). At a later stage, known as carcinoma *in situ*, these cells acquire a full malignant phenotype, except the ability to invade the surrounding parenchyma through the basal membrane. Nonetheless, in its final phase the carcinoma cells break through the basal membrane and become an invasive carcinoma (Fig. 1.27). Breast cancer progression is a complex process. Clinical evidence has shown that, even pre-malignant lesions, which are closest in similarity to invasive carcinoma, may progress to invasive carcinoma. However, it has been estimated that less than 50-60% of these will progress to invasive carcinoma (Erbas, *et al.*, 2006). Hence, atypical hyperplasia and carcinoma *in situ* are non-obligate precursors of breast cancer. Since breast cancer originates in normal breast epithelium it can be suggested that stimuli that normally result in breast gland proliferation will also boost the growth of breast cancer cells, intrinsically characterised by their proliferation advantage (Visvader, 2009). Mammary gland epithelium proliferation presents a marked dependence on hormones (Brisken, *et al.*, 2010, Macias, *et al.*, 2012). This way, glandular units of the female breast present a

cyclic growth and atrophy throughout the menstruation cycle, with the most marked hyperplasia during pregnancy and lactation, and regression during menopause (Russo*, et al.*, 2014).

Since estrogen is of great importance in breast epithelium growth and E2 interacts directly with ER to direct cell biology, it can be deduced that most mammary epithelial cells are ERα positive at one point in time (Allred*, et al.*, 2001, Clarke*, et al.*, 1997). Yet in malignant conversion through atypical hyperplasia and carcinoma *in situ* towards invasive breast cancer (Shoker*, et al.*, 1999), the ER positivity is increased, with around two thirds to three quarters of ductal carcinoma *in situ* and invasive breast cancers showing positivity (Li*, et al.*, 2003). Interestingly, a significant minority of cases of ER+ve breast cancers can be explained by an ESR1 (ERα) gene amplification (Holst*, et al.*, 2007). This suggests that for a majority of breast cancers, high ERα expression is a result of other mechanisms such as post-translational modifications.

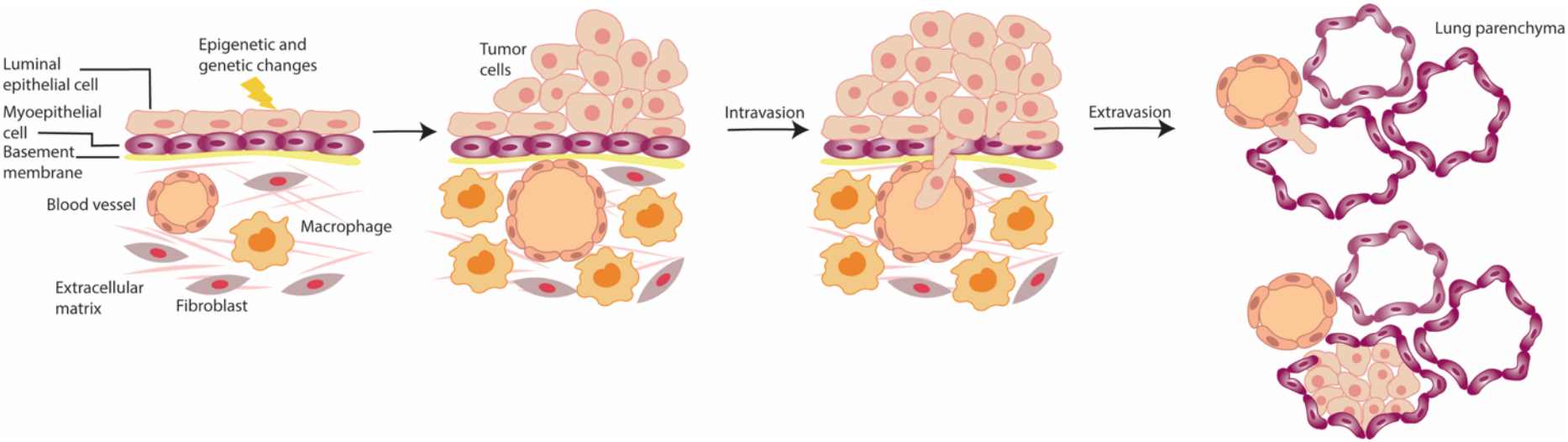


Figure 1.27: A schematic representation of breast cancer progression. The transformation of breast epithelial cells gives rise to metastatic breast cancer. During this multistage process, control of proliferation, survival, differentiation and migration become deregulated, and aberrant tumour-stromal cell interactions facilitate this process. To form metastases, cells must invade through the basement membrane, survive in the absence of adhesion and establish a new tumour in a foreign microenvironment (from (Vargo-Gogola*, et al.*, 2007) with permission).

1.5.2. Staging of Breast Cancer

The Union for International Cancer Control (UICC) categorises cancer into four stages based on the size of the tumour and the extent of spread. Stage 1 is small and confined within the breast. Stage 2 usually means the cancer has not spread to the lymph nodes close to the tumour. Stage 3 is a larger cancer with some spread to the surrounding tissue and lymph nodes. Stage 4 is the spread of the cancer to other organ/s. Advanced disease is defined as stages 3 and 4. In addition, breast cancer is further classified by the cellular expression of human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR) and ERs (Yersal*, et al.*, 2014). This is then used to identify appropriate treatments.

The high incidence, complexity, and the economic cost of treatment for breast cancer make it one of the most relevant health problems in our society today. Wide-spread population-based screening programs implemented in Western countries over the last 30 years have allowed the diagnosis of the disease at earlier stages, which has led to reduced mortality. However, since the establishment of screening programs there has been debate over the magnitude of the harm (e.g. overdiagnosis) that may come from screening. For example, the consequence of overdiagnosis is that women have their cancer treated by surgery, and in many cases medication and radiation, but neither women nor her doctor can know whether that particular cancer would have become apparent in the absence of screening (Marmot*, et al.*, 2013). In addition, treatments such as surgery, radiotherapy, chemotherapy, and the development of hormonal treatment, have significantly improved. Tamoxifen has significantly improved quality of life and often extends a patient's life for up to 20 years (Davies*, et al.*, 2017).

Substantial advances have been made in the treatment and detection of breast cancer, however, the age-standardised incidence of breast cancer still continues to increase in some countries (e.g. U.S.A.) (DeSantis*, et al.*, 2015). This age-standardised incidence is projected to continue to rise for these countries over the next 20 years. Clearly, there is still significant risk women are exposed to that is not being addressed.

1.5.3. Breast Cancer Risk Factors

Since the incidence of breast cancer increases with increasing age, this can give the impression that it is a disease of predominantly middle-aged and older women. In a sense that is true, indeed for the clinical part of the disease. However, it means that early life exposures must also be explored (i.e. at puberty and adolescence) to understand risk. From a breast cancer perspective, the 'lifecycle' of the breast can be divided into five windows of vulnerability: in utero, pubertal, pregnancy, postpartum involution and age-related involution (Fig. 1.28), representing identified times of risk throughout a woman's life. The unique biology of the breast begins in utero with breast ductal anlagen development. After birth, the ductal architecture grows slowly before ductal side branches and alveoli ensue during puberty, filling the mammary fat pad, poised to respond to pregnancy hormones. Pregnancy drives alveolar proliferation and differentiation and terminal differentiation of the gland with full term pregnancy and lactation. Post-partum, when lactation has ceased, the mammary gland undergoes involution where it remodels to a state morphologically and functionally similar to pre-pregnancy. Finally, with declining ovarian function, the mammary gland begins age related involution during peri-menopause where alveolar lobules regress. Involution is complete when a woman has gone through menopause and the parenchyma disappears (Fig.1.28) (Adami*, et al.*, 1995, Brisken*, et al.*, 2010, Colditz*, et al.*, 1995, Hilakivi-Clarke*, et al.*, 2006, Milanese*, et al.*, 2006, Pike*, et al.*, 1983, Schedin, 2006). Interestingly, many first clinical breast cancers appear when breasts begin the involution process (Russo, 2016). Therefore, the risk of developing breast cancer later in life is likely to increase during these lifecycle windows. In addition, nulliparity (i.e. not having children) also increases breast cancer risk with no disruption of age-related involution(Lyons*, et al.*, 2009, Oh*, et al.*, 2017).

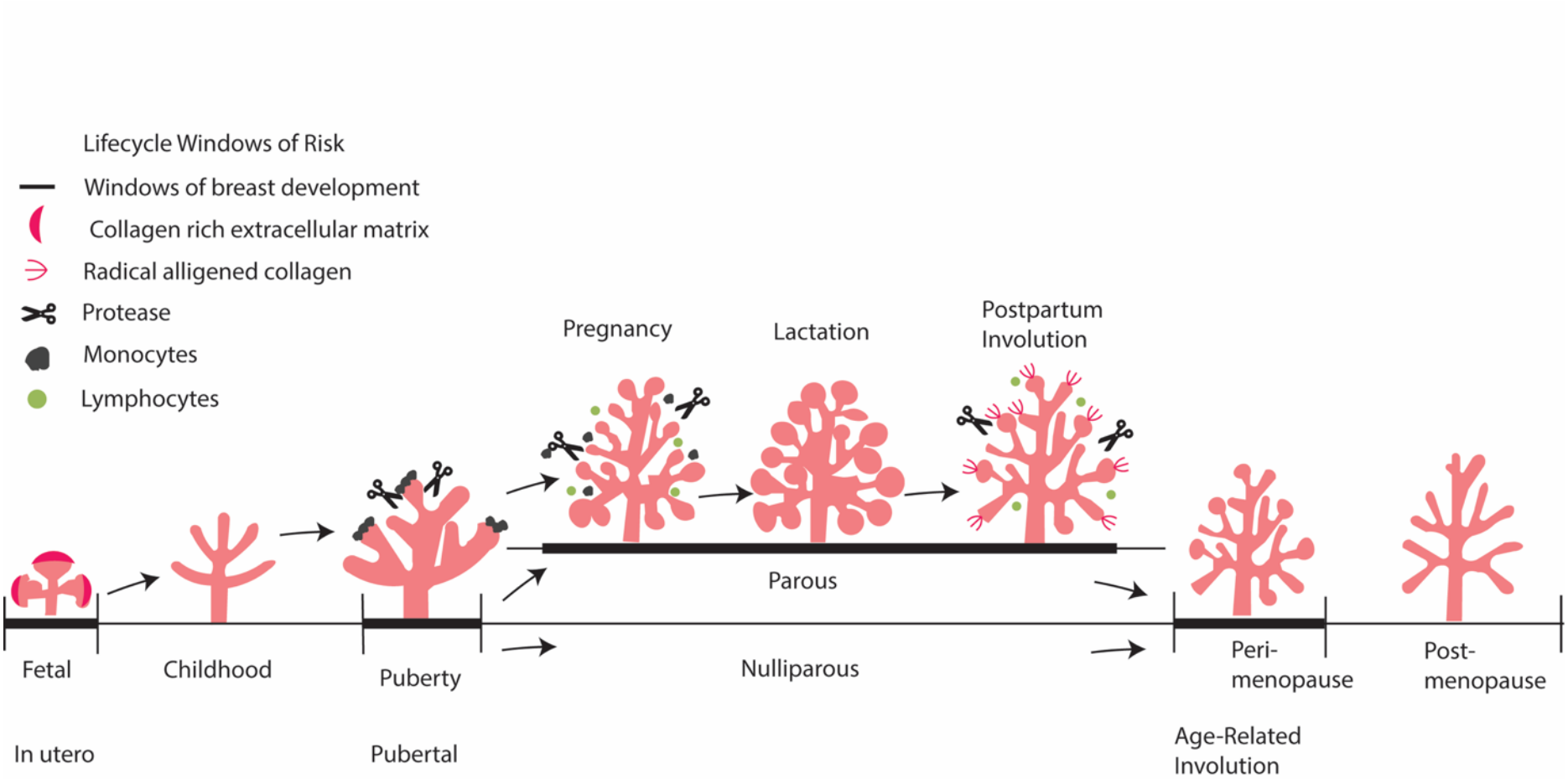


Figure 1.28: Lifecycle windows of risk for breast cancer. Schematic presentation of the lifecycle of breast development in women. The pregnancy, lactation, and involution cycle of the breast is offset to distinguish parous from women who have never been pregnant (nulliparous). The four windows of cancer vulnerability are defined by bold black lines: fetal, puberty, postpartum involution, and age-related involution (from (Martinson*, et al.*, 2013) with permission).

After sex and age, familial predisposition is the most important risk factor for breast cancer, however, not even 10% of the breast cancer patient population has an inherited germ line gene mutation. Germ line mutations of the breast cancer susceptibility genes, BRCA1 and BRCA2, are well known and responsible for a large part of the familial breast and ovarian cancer syndrome. A child born with a germ line mutation in BRCA1 has a 70-80% lifetime risk of developing breast cancer. For BRCA2, the penetrance is somewhat less but still amounts to almost 60% (Rebbeck*, et al.*, 2015). Most BRCA1 cancers have the basal cell phenotype, whereas this phenotype is rarely found in BRCA2 carcinomas that tend to be ER+/PR+ (Honrado*, et al.*, 2005).

Exposure to estrogens, whether endogenous or exogenous, is the main determinant of breast cancer risk. They have a dual effect: direct hormone action and genotoxicity. The prevailing model proposes that estrogens increase the rate of cell proliferation by stimulating ER mediated transcription, and thereby the number of errors occurring during DNA replication (Martinez-Ramirez*, et al.*, 2013).

An alternative hypothesis proposes that estrogens can be metabolised into quinone derivatives which react with DNA and then remove bases from DNA via depurination. The error prone DNA repairs then result in point mutations. Interestingly, metabolism of diethylstilbestrol, (a synthetic estrogen formerly used to reduce the risk of miscarriage and premature labour in pregnancy), causes high production of quinone derivatives compared to E2, whereas EE2 produces fewer quinone derivatives compared to E2 (Zhu*, et al.*, 1993). Phytoestrogens in general seem to form more 2-catechol estrogens (CE), which results in lower quinone production. Soy products are even able to change estrogen metabolism to less toxic CE, but the effects are presumably much more complex (Lehmann*, et al.*, 2008).

Age

The majority of breast cancer cases occur among women aged 50 years or older. However, according to the New Zealand Ministry of Health, about 1 in 10 breast cancers develop in women younger than 45 and about 1 in 2 breast cancers are found in women between 45-64 years (MOH, 2015). Age is not a unique risk factor for breast cancer, it is a risk factor for most diseases. This is due to the fact that the longer a person lives, the more opportunities there are for genetic mutations, and the body becomes less capable of repairing genetic damage.

Exogenous estrogens (HRT)

Current or recent users of hormonal replacement therapy (HRT) have a higher risk of developing breast cancer. Many postmenopausal women took HRT to alleviate postmenopausal symptoms such as hot flashes and fatigue. However, since the link between HRT and breast cancer was established the number of women taking HRT has dropped dramatically (Ravdin, *et al.*, 2007). There are two types of HRT available: combination HRT (containing both estrogen and progesterone) and estrogen-only HRT. Current users of combination HRT approximately double their breast cancer risk (Rossouw, *et al.*, 2002). In contrast, current users of estrogen-only HRT have a 30% increase in risk. The risk increases with duration of use, but declines rapidly after cessation of HRT use (Banks, *et al.*, 2003, Chlebowski, *et al.*, 2003, Colditz, *et al.*, 1995, Ross, *et al.*, 2000, Rossouw, *et al.*, 2002, Schairer, *et al.*, 2000). In addition, HRT greatly increases the risk of uterine cancer; thus, estrogen-only preparations are prescribed women who has had a hysterectomy (Beral, *et al.*, 2005).

Environmental risk

While there have been substantial advances in understanding breast cancer risk factors, especially the role of estrogens and genetics, for a majority of women presenting with breast cancer it is not possible to identify specific risk factors (IARC, 2008, Lacey, *et al.*, 2009). However, it is thought that increasing adoption of the Western lifestyle may be adding to breast cancer risk around the world. Interestingly, women who have migrated from China to the U.S.A have an increased risk of developing breast cancer, approaching 80% to that of the risk in the U.S.A. The daughters of those migrants will have a breast cancer risk that is almost identical to that of women who have lived in the U.S.A for generations (Ziegler, *et al.*, 1993). Clearly, the environment is a major component of breast cancer risk.

Alcohol and exercise

Research has consistently shown an association between alcohol consumption and breast cancer risk. It has been shown that women who have three alcoholic drinks per week have a 15% higher risk of developing breast cancer (McDonald, *et al.*, 2013). It is also known that physical activity of between 4-7 hr a week lowers breast cancer risk. This is attributed to exercise consuming and controlling blood sugar which in turn limits blood levels of insulin-like growth factor (IGF), a hormone that affects how breast cells grow and behave (Xie, *et al.*, 2013). Therefore, people that do not exercise regularly have an increased risk of developing breast cancer.

Xenoestrogens

Currently the contribution xenoestrogens play in breast cancer risk is debatable. However, an elevated incidence of breast cancer in women has been associated with prolonged exposure to high levels of estrogens, especially pre-menarche and post-menopausally (Bernstein, *et al.*, 1993, Henderson, *et al.*, 1991). However, exposure to xenoestrogens is not limited to these two stages in a woman's life, in fact, exposure can occur throughout a woman's life (Soto, *et al.*, 2007).

1.6 Model Systems for Studying Xenoestrogen Responses

There are many model systems that can be used to study xenoestrogens. One is a breast cancer cell line (MCF-7) that expresses both the ERα and ERβ, another model system is a reporter gene assay (e.g. Chemically activated luciferase expression (CALUX®) and another is a computational model system, namely Schrodinger. These are described in detail below.

1.6.1. The MCF-7 Model System

The MCF-7 cell line was first isolated in 1970 from a 69-year-old Caucasian woman who had breast cancer. The cell line has been used extensively for research, as it was the first mammalian cancer cell line capable of surviving in culture for longer than a few months. MCF-7 cells, in addition to their longer life span, were the first ER-positive (ER+) and estrogen responsive breast cancer cell line to be documented. These cells have been widely used in xenoestrogenic studies as they have both ERα and ERβ, allowing them to rapidly grow when exposed to E2 (Levenson, *et al.*, 1997). The MCF-7 model system, as mentioned above, has both ER isoforms and is a breast cancer cell line. This is the closest *in vitro* system that models a breast cell, therefore, when xenoestrogen exposure studies are carried out it is likely to give the most representative results. However, these cells can be difficult to manipulate, hence, other systems have also been developed.

1.6.2. CALUX® Reporter Gene Assay

CALUX® is a bioassay used as an effect-based screening method that is able to measure the total effect a ligand has on its receptor. The assay consists of a cell that has been genetically modified with a luciferase reporter gene, producing luciferase – a light generating enzyme. In gene reporter assays the DNA sequences containing the EREs are linked to the gene of an easily measurable protein, for example firefly luciferase. In this case, the ligand-activated receptor will activate luciferase transcription, and the transcribed luciferase protein will emit

light when a substrate is added (e.g. luciferin), see Fig. 1.29. The signal will dose dependently increase as a result of increasing concentrations of xenoestrogen, allowing precise quantification of *in vitro* hormone activity of compounds (Sonneveld*, et al.*, 2005).

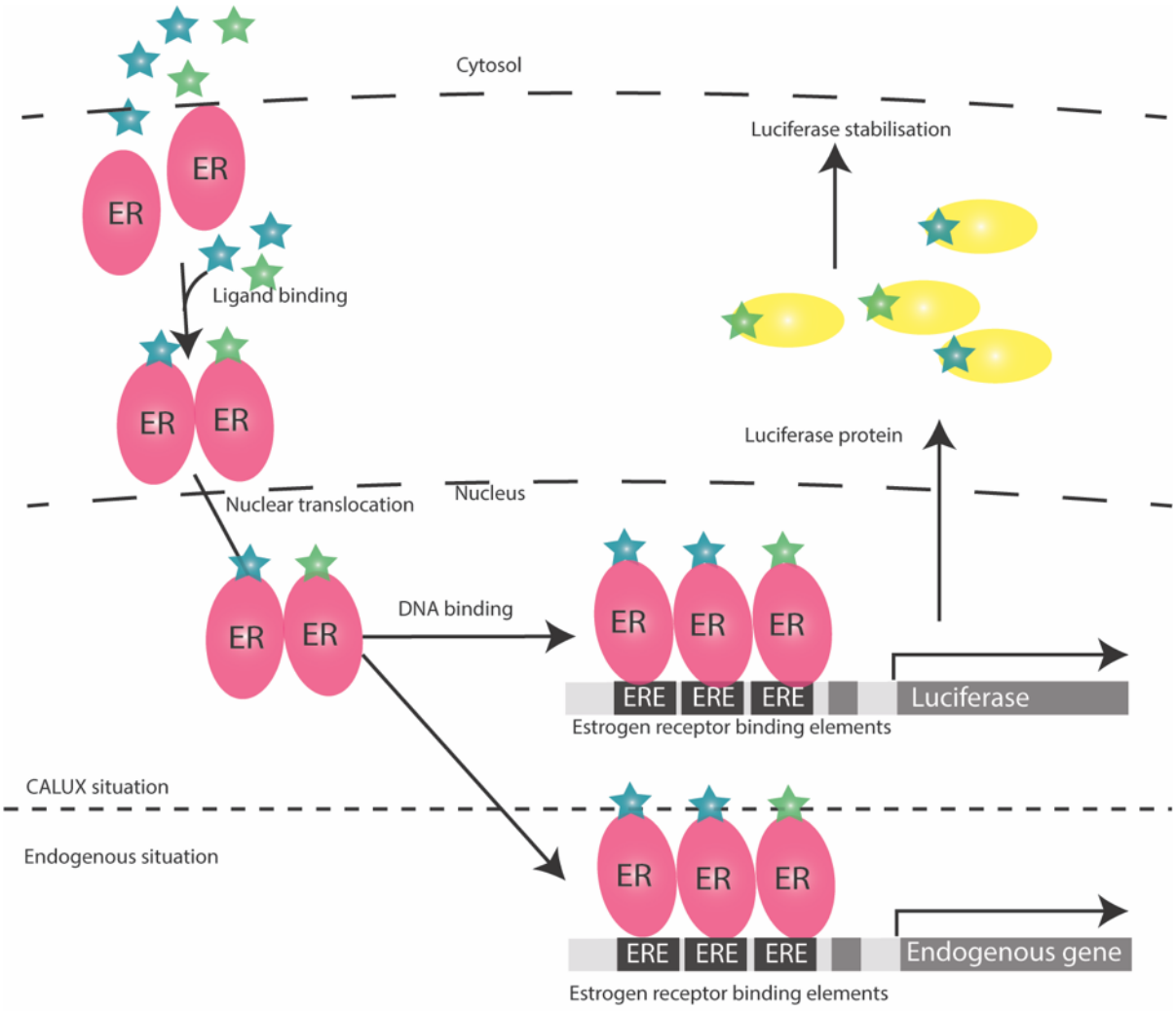


Figure 1.29: Principle of the CALUX® reporter gene assays. Estrogenic chemicals ( ), as a component of a cocktail, cross the cell membrane and bind to ERs. The ligand-bound ER dimer enters the nucleus, binds to DNA receptor binding elements, resulting in up-regulated luciferase and luciferase protein synthesis. The addition of luciferin (substrate) results in light production, where the number of photons produced is proportional to the estrogenicity of the xenoestrogen cocktail. In the endogenous situation, the molecular mechanism is identical, except other estrogen responsive genes are up-regulated since luciferase is not present (from Biodetection Systems Ltd with permission).

The ERα and ERβ CALUX® reporter gene assays are constructed in U2OS (human osteosarcoma) cells. The U2OS cells are an ideal cell to choose as they exhibit no or very low expression of endogenous receptors. Therefore, this assay requires the genetic modification to express luciferase and the desired receptor (e.g. ER α or β). The advantage of such a system is that cross-talk between various nuclear hormone receptors cannot occur, resulting in highly selective and responsive assays (Sonneveld*, et al.*, 2005). These assays have been proven to accurately predict estrogenic activity of chemicals and pharmaceuticals. Reporter gene assays have several advantages over classical *in vivo* assays for estrogenicity, not only by increasing speed and reduced costs but also reduced use of animals and the use of human cells and human receptors (Sonneveld*, et al.*, 2006, van der Burg*, et al.*, 2010). Furthermore, reporter gene assays are ideally suited to accurately and quantitatively measure the combined effect exerted by all chemicals present in complex mixtures (van der Burg*, et al.*, 2013). However, it is a more simplified method for studying xenoestrogenic compounds and does not fully represent the *in vivo* situation. Therefore, it is important to also do the MCF-7 experiments to ensure the most accurate representation of a human breast cell is attained.

1.6.3. Schrodinger

Another model system is computational models. Schrödinger is an advanced computational modelling system where the docking of xenoestrogens can be simulated. It has been widely used in the study of xenoestrogens to explore the binding interactions of specific xenoestrogens with ERs (Celik*, et al.*, 2008, D'Ursi*, et al.*, 2005, Lambrinidis*, et al.*, 2006). Much of the computational docking done with the ER has been for drug development (Knox*, et al.*, 2006, Knox*, et al.*, 2008) for the treatment of breast cancer, osteoporosis and prostate diseases. Docking studies maybe able to shed light on the small local changes that occur upon ligand binding that are then amplified through the protein structure resulting in large conformational changes. Since the objective of this study was to use the tool to study the intimate interactions of xenoestrogens with the ERs, computational docking was used in conjunction with cell model systems to begin to predict the effects of xenoestrogens.

1.7. Goals and Objectives

- Characterise and perfect the MCF-7 cell culture system. Determine optimum growth conditions by observing MCF-7 growth characteristics following modification of culture media (e.g. changes in non-essential amino acids (NEAAs)). Determining optimum tryspin methodology to de-adhere cells from culture vessels.
- Investigate the effects of xenoestrogens and combinations of xenoestrogens on MCF-7 cells in culture by exposing cultured MCF-7 cells to varying concentrations of selected xenoestrogens both individually and in combinations and plotting growth curves.
- Investigate the estrogenic potency of individual and combinations of xenoestrogens on both ER isoforms (ERα and ERβ) of the CALUX® assay by exposing ERα and ERβ CALUX® cells to varying concentrations of selected xenoestrogens both individually and in combinations and determining EC_{50} values.
- Use *in silico* modelling (Schrödinger platform) to investigate the biomolecular interactions of xenoestrogens with ER ligand binding sites (LBC and AF-2) and explore the interplay between the two binding sites. Using ligand-receptor docking to study the biological chemistry of biomolecular interactions of selected xenoestrogens with ERs, and determine the importance of ligand structure in predicting interactions with LBC and AF-2.
- Assess women's and pre-pubertal girls' exposures to xenoestrogens using a dietary and lifestyle questionnaire, and determine the contribution of xenoestrogen exposure to the theoretical total estrogenic load.
- Determine serum markers of xenoestrogen exposure (e.g. BPA, genistein, butylparaben), based on women's exposures from the dietary and lifestyle questionnaire study, by taking blood samples from a group of women and determining serum levels of a selection of xenoestrogens by LC-MS.
- Bring together data from the cell culture, CALUX®, *in silico* and exposure studies to explore the relevance of xenoestrogens in a breast cancer risk context.

Chapter 2 Materials and Methods

2.1. Materials

A list of materials used in cell culture and blood analysis are presented in Table 2.1

Table 2.1: Materials used in cell culture and blood analysis

Material	**Supplier/model**	**Location**
Cell culture		
Chemicals		
β–Estradiol (E2)	Sigma-Aldrich	Auckland, NZ
Estrone	Sigma-Aldrich	Auckland, NZ
Estriol	Sigma-Aldrich	Auckland, NZ
17α-Ethinyl estradiol (EE2)	Sigma-Aldrich	Auckland, NZ
Bisphenol A (BPA)	Sigma-Aldrich	Auckland, NZ
Genistein	LC-Laboratories	Massachusetts, USA
Daidzein	LC-Laboratories	Massachusetts, USA
Kaempferol	Sigma-Aldrich	Auckland, NZ
Curcumin	Sigma-Aldrich	Auckland, NZ
Tetrahydrocurcumin	Sigma-Aldrich	Auckland, NZ
Methyl 4-hydroxybenzoate	Sigma-Aldrich	Auckland, NZ
Butyl 4-hydroxybenzoate	Sigma-Aldrich	Auckland, NZ
Benzyl 4-hydroxybenzoate	Sigma-Aldrich	Auckland, NZ
Streptomycin sulphate salt	Sigma-Aldrich	Auckland, NZ
Penicillin G sodium salt	Sigma-Aldrich	Auckland, NZ
Commercial phosphate buffered saline (PBS) packet (total weight = 9.55 g comprising sodium chloride (8 g), potassium phosphate, monobasic (0.2 g), sodium phosphate, dibasic (1.15 g) and potassium chloride (0.2 g))	Sigma-Aldrich	Auckland, NZ
Gibco MEM non-essential amino acids (NEAA)	Thermofisher Scientific	Christchurch, NZ
Analytical grade ethanol	ECP Ltd	Auckland, NZ
Sodium bicarbonate	ECP Ltd	Auckland, NZ
Ethylenediaminetetracacetic acid (EDTA)	ECP Ltd	Auckland, NZ
Ground dextran coated charcoal	Sigma-Aldrich	Auckland, NZ
Magnesium chloride hexahydrate	ECP Ltd	Auckland, NZ
Sucrose	ECP Ltd	Auckland, NZ
4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	ECP Ltd	Auckland, NZ

Trypan Blue	Sigma-Aldrich	Auckland, NZ
Tris	Sigma-Aldrich	Amsterdam, NL
Dithiothreitol (DTT)	Sigma-Aldrich	Amsterdam, NL
1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid (CDTA)	Sigma-Aldrich	Amsterdam, NL
EDTA	Sigma-Aldrich	Amsterdam, NL
Glycerol	Sigma-Aldrich	Amsterdam, NL
Triton®X-100	Sigma-Aldrich	Amsterdam, NL
Tricine	Sigma-Aldrich	Amsterdam, NL
$Mg(CO_2)_4Mg(OH)_2.5H_2O$	Sigma-Aldrich	Amsterdam, NL
$MgSO_4.7H_2O$	Sigma-Aldrich	Amsterdam, NL
Dextran T500	Sigma-Aldrich	Amsterdam, NL
Biological products		
RPMI-1640 powder	Life Technologies	Auckland, NZ
Gibco phenol red-free RPMI-1640	Life Technologies	Auckland, NZ
Fetal bovine serum	Life Technologies	Auckland, NZ
Trypsin powder	Sigma-Aldrich	Auckland, NZ
Gibco trpLE™ express	Life Technologies	Auckland, NZ
MCF-7 human breast cancer cells	American Type Culture Collection (ATCC)	Manassas, USA
ERα-CALUX® cells	BioDetection Systems	Amsterdam, NL
ERβ-CALUX® cells	BioDetection Systems	Amsterdam, NL
D-Luciferin	Sigma-Aldrich	Amsterdam, NL
ATP	Sigma-Aldrich	Amsterdam, NL
Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12)	ThermoFisher Scientific	Amsterdam, NL
Gibco Sodium pyruvate (1 M)	Life Technologies	Auckland, NZ
Equipment		
T-75 sterile cell culture flasks	In Vitro Technologies	Auckland, NZ
T-25 sterile cell culture flasks	Sigma-Aldrich	Auckland, NZ
Glass vials (4 mL, 7 mL and 20 mL)		
24 Well plate	Sigma-Aldrich	Auckland, NZ
White 384 well plate	Sigma-Aldrich	Amsterdam, NL
50 mL Centrifuge tubes		
Automatic pipette and tips		
Sterile filter (Steritop-GP, 0.22 µm, polyethersulfone, 500 mL 45 mm	ThermoFisher Scientific	Melbourne, AUS
Sterile syringe filter (17 mm, 0.2 µm PTFE filter)	ThermoFisher Scientific	Christchurch, NZ
Autoclave		
Laminar flow cabinet	Cytoguard CG2000 series, model CGA-180	Sydney, AUS
Inverted microscope	CKX41, Olympus	Melbourne, AUS
Microscope camera	ProSciTech	Kirwan, AUS
Toup camera software	ProSciTech	Kirwan, AUS
Veriplast plastic counting chambers	Thermo Fisher Scientific	Melbourne, AUS
Centrifuge	MultiFuge 1 S-R	Hanau, Germany
Glassware		
Hot-plate stirrer		

Microwell plates (24, 384 wells)	ThermoFisher Scientific	Auckland, NZ
Schott bottles (100 mL, 500 mL, 1000 mL)		
Cytomat Incubator	ThermoFisher Scientific	Amsterdam, NL
Hamilton Robot	STARlet	Bonaduz Switzerland
EL406 Washer-Dispenser	BioTek	Amsterdam, NL
Luminometer	Tecan	Mannedorf, Switzerland
Blood analysis		
Chemicals		
Analytical grade acetonitrile	ECP Ltd	Auckland, NZ
Analytical grade Methyl *tert*-butyl ether	Sigma-Aldrich	Auckland, NZ
Analytical grade chloroform	ECP Ltd	Auckland, NZ
Sodium sulphate	ECP Ltd	Auckland, NZ
Equipment		
Sterile needles	Becton Dickinson	Auckland, NZ
Vacutainer	Becton Dickinson	Auckland, NZ
50 mL Centrifuge tubes		
Centrifuge	MultiFuge 1 S-R	Hanau, DE
High performance liquid chromatography (HPLC) vials	Micro-Analytix	Auckland, NZ
Whatman filter paper	ThermoFisher Scientific	Auckland, NZ
Glassware		
Speed Vac	ThermoFisher Scientific	Auckland, NZ
Glass vials		
LC-MS		
C18 reverse phase HPLC column	Phenomex	North Shore, NZ

2.1.1 MilliQ Water

All water used in MCF-7 and Blood analysis experiments was purified using the Milli-Q® system (Merck Millipore, Auckland, New Zealand). Resistivity (25°C) = 18.2 MΩ.cm.

2.1.2. Demineralised Water

All water used in CALUX® assay experiments was purified using a HOH RO 51 Reverse Osmosis Plant from HOH Water Technology (Greve, Denmark). Water quality = 40 µS/cm (0.25 MΩ.cm).

2.1.2. Computational Modelling

2.1.2.1. Receptor Models

Computational docking relies on models of the receptor protein structure. These models are developed from published x-ray crystal structures. For this work, the x-ray crystal structures of the ER LBD (Table 2.2) were obtained from the RSCB Protein Data Bank (PDB; http://www.rcsb.org). The models developed from the crystal structure are identified by the PDB code.

Table 2.2: X-ray crystal structures of the human ER ligand binding domains obtained from the PDB.

PDB code	Receptor	Ligand	Resolution (Å)	Chain	Coregulatory protein peptide	Reference
1ERE	ERα	E2	3.10	A	no	(Brzozowski, *et al.*, 1997)
3OLS	ERβ	E2	2.20	A	yes	(Mocklinghoff, *et al.*, 2010)

2.1.2.2. Ligand Structures

Computational docking requires a model for each ligand which is developed from their 3D structures. The 3D structures for all ligands were constructed using the software tools (see Section 2.2.10.1.) from the Schrodinger Suite. Models of 16 xenoestrogens (estrone, E2, estriol, progesterone, testosterone, genistein, daidzein, equol, kaempferol, curcumin, tetrahydrocurcumin, EE2, BPA, methylparaben, butylparaben and benzylparaben (see Figs. 1.1 and 1.19) were required for the study.

All ligands were energy minimised using the Schrödinger software to achieve an optimised conformation of the ligand that had the lowest internal energy. This calculation searched the entire conformation space of the ligand, including ring conformations (e.g., 6-member ring boat/chair conformations) and rotational degrees of freedom. For the ring conformation search, only ligands with minimised energy within 50 kJ/mol of the lowest energy conformation were retained. For most ligands only one conformation was found that fitted this parameter.

2.2. Methods

MCF-7 culture studies

2.2.1. Sterilisation

2.2.1.1. Glassware and Consumables

All glassware and consumables including glass pipettes, micropipettes, Pasteur pipettes, Schott bottles and Eppendorf tubes were autoclaved at 120 °C, 15 psi for 80 min.

2.2.1.2. Maintaining an Aseptic Work Surface

All cell culture procedures were conducted in a laminar flow cabinet with an internal work surface pre-sterilised by UV radiation (254 nm, 2 h). Immediately prior to any cell culture work, the internal surface was sterilised with 70% v/v ethanol aerosol. All equipment and reagents were sprayed with ethanol aerosol and immediately transferred inside the laminar flow cabinet.

2.2.2. Preparation of Cell Culture Media and Related Reagents

2.2.2.1. Preparation of Phosphate Buffered Saline (PBS)

A packet of PBS powder was added to a 1 L Schott bottle. MilliQ (900 mL) was added and the PBS dissolved by vigorous shaking. The pH was adjusted to 7.4 using 1 M HCl or NaOH as appropriate. The pH adjusted solution was topped up to 1 L with MilliQ water. The solution was sterilised via ultra-filtration through a 0.22 μM filter and stored at 4°C.

2.2.2.2. Preparation of Antibiotics for Addition to Culture Media

Benzyl penicillin (3.0 g) and streptomycin (2.8 g) were made up to 100 mL of sterile MilliQ water and stirred using a magnetic stirrer for 24 h. The solution was stored at 4°C for up to 6 months.

2.2.2.3. Preparation of Trypsin Protease

NaCl (8.5 g) was dissolved in MilliQ water and made up to 1 L. Trypsin powder (25 g) was added to the 0.85% (w/v aq) NaCl (1 L) and stirred (using a magnetic stirrer) at room temperature for 1 h. The trypsin solution was sterilised by syringe filtration and dispensed into 10 mL aliquots and stored at -20°C. EDTA (3.72 g) was dissolved in PBS (1 L) and sterilised by filtration. This PBS/EDTA (PE) solution was diluted 10-fold with PBS and 90 mL of the diluted PE was mixed with trypsin solution (10 mL) to produce the final trypsin solution (2.5% aq). This solution was stored at 4°C for up to 3 weeks.

2.2.2.4. Heat Inactivation of FBS

FBS (500 mL) was thawed at 4°C overnight. The thawed serum was gently warmed in a 37°C water bath for 30 min with gentle inversion every 10 min to ensure even temperature distribution. After the serum reached 37°C it was placed in a 56°C water bath for 60 min with gentle inversion every 10 min. The serum was left to rest at room temperature for 30 min. Aliquots (100 mL) were transferred to Schott bottles and stored at -20°C.

2.2.2.5. Preparation of Charcoal-dextran Stripped FBS

$MgCl_2(H_2O)_6$ (0.3 g), sucrose (85.6 g), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 2.4 g) was added to a 1 L Schott bottle. MilliQ water (1 L) was added to the bottle and shaken vigorously. The solution was divided into 50 mL aliquots. Two aliquots were added to two 50 mL plastic centrifuge tubes each containing dextran coated charcoal (0.137 g). The centrifuge tubes were inverted 10 times to ensure even distribution of charcoal-dextran. The tubes were incubated for 24 h at 4°C. Following incubation, the charcoal-dextran solution was centrifuged at 500 xg for 10 min and the supernatant was discarded. Inactivated FBS (50 mL) was added to each pellet. The tubes were inverted 10 times and incubated for a further 24 h at 4°C. The FBS-charcoal solution was then centrifuged at 1700 xg for 10 min and the stripped FBS decanted into a 100 mL Schott bottle and stored at -20°C.

2.2.2.6. Preparation of Phenol Red RPMI-1640 Medium Containing 10% v/v Inactivated FBS

Heat inactivated FBS was thawed at 4°C overnight. Phenol red RPMI-1640 powder (9.6 g) and $NaHCO_3$ (2.0 g) were added to a 1 L Schott bottle. MilliQ (900 mL) was added and the powder dissolved via vigorous shaking. The pH was adjusted to 7.4 (see Section 2.2.2.1.) and MilliQ (100 mL) was added. Sodium pyruvate solution (1 M; 1 mL) was added to the RPMI-1640 solution and 900 mL was sterilised via ultra-filtration through a 0.22 µM Millipore filter. Ultrafiltration sterilised anti-biotics (5 mL) and FBS (100 mL) were added in that order to the RPMI-1640 culture medium. The complete RPMI-1640 culture medium solution (1 L) was stored at 4°C.

2.2.2.7. Preparation of Phenol Red Free RPMI-1640 Medium Containing 10% v/v Stripped FBS

Charcoal-dextran stripped FBS (100 mL) was thawed at 4°C overnight. Gibco phenol red free (PRF) RPMI-1640 culture medium solution (900 mL) was sterilized via ultra-filtration using a 0.22 μM Millipore filter, followed by antibiotics (5 mL) and FBS (100 mL), respectively and stored at 4°C.

2.2.3. Cell Maintenance and Passaging

2.2.3.1 MCF-7 Cell Seeding

A 1 mL cryo-vial containing frozen MCF-7 cells was removed from storage in liquid nitrogen (-196°C) and thawed at room temperature for 15 min. Phenol red RPMI-1640 (5 mL) was added to a sterile 25 cm^2 culture flask using a flame sterilised 10 mL glass pipette. The defrosted cell suspension (100 μL) was added to the RPMI-1640 using a micro pipette with a sterile tip. The cells were incubated at 37°C in 5% v/v CO_2 until they reached confluence (approx. 5 days). When confluent, the cells were passaged into sterile 75 cm^2 culture flasks as described in Section 2.2.3.2.

2.2.3.2. Maintenance of MCF-7 Cell Culture

MCF-7 cells were routinely passaged when the cultures reached at least 70% confluence (approx. 10^7 cells). Confluence refers to the percentage of the surface of a culture flask that is covered by adherent cell, in this case a 70% coverage was required before routine passage. Spent phenol red RPMI-1640 culture medium was vacuum aspirated using a flame sterilised Pasteur pipette. PBS (4 mL) was added to wash away any residual RPMI-1640 culture medium from the flask. Gibco TrpLE® express (a proprietary trypsin solution, 3 mL) was added to detach the cellular monolayer. The culture flasks were incubated at 37°C with 5% v/v CO_2 until the cells were seen to be beginning to detach under an inverted microscope. RPMI-1640 (6 mL) was added to inactivate the TrpLE® express and the cell suspension was transferred to two new sterile 75 cm^2 culture flasks. Further RPMI-1640 (12 mL) was added to each flask to give a total volume of 18 mL. The cell cultures were incubated at 37°C (see Section 2.2.3.1.).

2.2.4. Cell Counting

2.2.4.1. Vetriplast® Plastic Counting Chambers

Vetriplast® Plastic Counting Chambers were used to count large volumes of cells samples at any given time. A 20 µL aliquot from each sample of cell suspension was collected from a known volume and mixed 1:1 with trypan blue. A 9 µL aliquot of each sample was added to the 9 x 9 grids (10 per chamber). The same 5 squares were counted for each of the 10 samples under an inverted microscope at 100x magnification. The total cell number was calculated from the formulae:

$$C_{mL} = \frac{T \times 10^3}{k \times N} \qquad C_{total} = C_{mL} \times V$$

- $C_{mL} = Cells\ per\ mL$
- $C_{total} = Total\ number\ of\ cells\ in\ the\ cell\ suspension$
- $T = Total\ number\ of\ cells\ counted$
- $k = 0.1111$
- $N = 5$
- $V = Total\ volume\ of\ cell\ suspension$

2.2.5. MCF-7 Exposure Experiments

Exposure experiments were used to determine the exposure concentration of the combinations of xenoestrogens to be used in the MCF-7 growth curve experiments (see Section 2.2.6.). A confluent flask was removed from incubation and the cells detached using 4 mL of 2.5% trypsin solution and incubated for 5-10 min. Fresh PRF-RPMI-1640 (10 mL) was added to each flask. The cell suspension from the flask was transferred to a 50 mL centrifuge tube and vortex mixed until a homogenous suspension was produced. Three 20 µL aliquots were removed for counting (see Section 2.2.4.2.), the remaining cell suspension was kept on ice to prevent cell aggregation. Once the total number of cells were determined, they were seeded into a 24 well plate and diluted to ensure a final concentration of 10^5 cells in each well. Fresh medium with the desired exposure compound/s was added to give a final volume of 2 mL of medium/well in a series of eight serial dilutions. The plate was incubated at 37°C with 5% v/v CO_2 for five days and counted in triplicate.

2.2.6. MCF-7 Growth Curve Experiments

Growth curve measurements were carried out in triplicate over 10 days. Confluent cells (3 flasks) were removed from incubation and the cells detached, vortex mixed, counted and seeded into a 24 well plate (see Section 2.2.5.). Fresh media was added to give a final volume of 2 mL of media/well. The plates were incubated at 37°C with 5% v/v CO_2. The 24 wells were counted in triplicate on days 1, 3, 5-8 and 10.

2.2.7. Cryopreservation

A flask of confluent MCF-7 cells was removed from incubation. The monolayer was detached (See Section 2.2.3.2.) and transferred to a 50 mL centrifuge tube. The suspension was centrifuged at 4000 xg for 5 min and the supernatant was vacuum aspirated. The pellet was suspended in phenol red RPMI-1640 containing 17% v/v DMSO (100 µL). Aliquots (100 µL) were added to sterile 1 mL cryovials. Each cryovial was wrapped in cotton wool and placed at -80°C for 24 h before being stored in liquid nitrogen (-198°C).

CALUX® Studies

2.2.8. Preparation of Cell Culture Media and Related Reagents

2.2.8.1. Preparation of Phosphate Buffered Saline (PBS)

A packet of PBS powder was added to a 1 L Schott bottle. MilliQ water (900 mL) was added and the PBS dissolved by vigorous shaking. The pH was adjusted to 7.4 (as described in Section 2.2.2.1.). The pH-adjusted solution was topped up to 1 L with MilliQ water. The solution was sterilised using ultra-filtration through a 0.22 µM Millipore filter and stored at 4°C.

2.2.8.2. Preparation of Trypsin Protease

Trypsin stock solution was diluted to a concentration of 0.05% using PBS (without calcium and magnesium) containing 0.2 g/L ETDA. The solution was sterilised using ultra-filtration through a 0.22 µM Millipore filter, aliquoted (40 mL) and stored at -20°C for up to three months.

2.2.8.3. Preparation of Cell Lysis Buffer

Demineralised water (500 mL) was added to a 1 L glass beaker. Tris, DTT and CDTA (see Table 2.3), glycerol (100 mL) and Triton®X-100 (10 mL) were added to the beaker and adjusted to pH 7.8 (see Section 2.2.2.1.). The final volume was adjusted to 1 L with demineralised water and aliquoted (40 mL) into 50 mL centrifuge tubes. The tubes were stored at -20°C for up to 1 year or 4°C for up to 1 month.

Table 2.3: Composition of cell lysis mix

Compound	Weight (g)	Volume (mL)	Molarity
Tris	3.0		25 mM
DTT	0.31		2.0 mM
CDTA	0.73		2.0 mM
Glycerol		100	10%
Triton®X-100		10	1%

2.2.8.4. Preparation of BioDetection Systems Illuminate Mix

Tricine and magnesium hydroxide carbonate pentahydrate (as per Table 2.4) were added to demineralised water (500 mL) and stirred using a magnetic stirrer until the solution was clear (approx. 1 h). Illuminate was warmed to room temperature and added along with magnesium sulphate-heptahydrate, EDTA and DTT (as per Table 2.5) to the solution in a dark room to prevent illuminate mix degradation. ATP (as per Table 2.5) and demineralised water (400 mL) was added and the solution was adjusted to pH 7.8 (see Section 2.2.2.1.). The final volume was adjusted to 1 L with demineralised water and mixed carefully. Aliquots (100 mL) were transferred into Schott and stored at -20°C for up to 3 months or at -80°C for up to a year.

Table 2.4: Composition of illuminate mix

Compound	Weight (g)	Molarity
Tricine	3.580	20.0 mM
$(MgCO_2)_4Mg(OH)_2.5H_2O$	0.520	1.07 mM
$MgSO_4.7H_2O$	0.658	2.67 mM
EDTA	0.037	0.100 mM
DTT	0.231	1.50 mM

D-Luciferin	0.151	0.539 mM
ATP	3.026	5.49 mM

2.2.8.5. Preparation of Dextran-Coated Activated Charcoal

Tris (0.6 g) was dissolved in demineralised water (500 mL) in a glass beaker and adjusted to pH 8.0 (see Section 2.2.2.1.). A further 450 mL of demineralised water was added to the Tris solution. Dextran T500 (0.25 g) and activated charcoal (2.5 g) were added to the buffer and stirred using a magnetic stirrer at 4°C overnight.

2.2.8.6. Preparation of Charcoal-dextran Stripped FBS

FBS (500 mL) was thawed and 200 mL aliquoted into a glass bottle by sterile pipetting prior to refreezing. The remaining FBS (300 mL) was gently warmed in a 56°C water bath for 30 min. Dextran-coated charcoal suspension (950 mL) was divided equally between 12 centrifuge tubes (50 mL) and centrifuged at 1000xg for 10 min. The supernatant was discarded leaving behind dextran-coated charcoal pellets. FBS was divided equally into 6 centrifuge tubes and incubated in a 45°C water bath for 45 min with continuous shaking. The serum-charcoal solution was centrifuged at 1000 xg for 20 min. The serum was decanted into the remaining 6 centrifuge tubes and incubated again at 45°C for 45 min and centrifuged at 1000 xg for 20 min. The stripped FBS was decanted into six clean 50 mL centrifuge tubes and centrifuged at 1000 xg for a further 20 min. Stripped FBS was pooled, sterilised by ultra-filtration and aliquoted (26.4 mL) into 50 mL centrifuge tubes and stored at -20°C for up to 6 months.

2.2.8.7. Preparation of DMEM/F12 Phenol Red Free Medium Containing 5% v/v Stripped FBS

FBS (41 mL) was thawed in a 37°C water bath and added with Gibco non-essential amino acids (NEAAs; 5.5 mL) and penicillin-streptomycin solution (1 mL; see Section 2.2.2.2.) to 500 mL of PR-DMEM/F12 culture medium and stored at 4°C.

2.2.9. Cell Maintenance and Passaging

2.2.9.1. Maintenance of U2OS CALUX® Cell Culture

U2OS CALUX® cells were routinely passaged when cultures reached at least 70% confluence (see section 2.2.3.2). Spent DMEM/F12 medium was removed from the flask and the cells were washed using PBS (5 mL) twice to inactivate any residual DMEM/F12. Trypsin (2 mL) was added to the flask to just cover the cells and then decanted before incubating the cells at 37°C with 5% v/v CO_2 until the cells were seen to begin to detach under an inverted microscope (approx. 1-3 min). DMEM/F12 (10 mL) was added to inactivate the trypsin and the cell suspension was transferred into four new sterile 75 cm^2 culture flasks. Further DMEM/F12 (7 mL) was added to each flask and the cells incubated at 37°C until confluent.

2.2.9.2. ERα and ERβ CALUX® Exposure Studies

The response of CALUX® to xenoestrogen combinations to assays was determined and repeated in triplicate. For both the ERα and ERβ CALUX® assays, cells were seeded into white 384-well plates at a density of 3000 cells/well and allowed to attach for 24 h. The Assay medium was removed from each well using an EL406 washer/dispenser (BioTek) and exposure medium applied with the desired xenoestrogen/s. To get the required combinations, ERα or ERβ CALUX® cells were exposed to increasing concentrations of each of the test compounds at a final DMSO concentration of 0.1% v/v, and in the 'background' a second compound was added at fixed concentration ([DMSO] = 0.01% v/v), giving an overall [DMSO] = 0.11% v/v. The fixed concentration was always around the EC_{10} concentration of the particular compound. Additionally, two DMSO blanks and a full dose response curve of the relevant reference compound (e.g. E2) were included on each plate. The preparation of the compound dilution series (e.g. the second exposure mixture) was performed on a Hamilton Starlet liquid handling robot coupled to a Cytomat incubator. After another 24 h, the medium was removed and Triton® X-100 lysis buffer (10 µL/well) was added using the BioTek EL406 washer/dispenser. Finally, illuminate mix was added to each well and the light signal was measured in a luminometer (TECAN Infinite Pro) for 0.017 min/well (i.e. ~1 s).

2.2.9.3. Cytotox CALUX® Studies

The response of the Cytotox CALUX® assay to selected xenoestrogen combinations were determined. Cells were seeded into white 384-well plates at a density of 3000 cells/well and allowed to attach for 24 h. The Assay medium was removed from each well using an EL406

washer/dispenser (BioTek) and exposure medium applied with the desired xenoestrogen/s. To get the required combinations, Cytotox CALUX® cells were exposed to increasing concentrations of each of the test compounds at a final DMSO concentration of 0.1% v/v, and in the 'background' a second compound was added at fixed concentration ([DMSO] = 0.01% v/v), giving an overall [DMSO] = 0.11% v/v (as per Section 2.2.9.2). Additionally, two DMSO blanks were included on each plate. The preparation of the compound dilution series (e.g. the second exposure mixture) was performed on a Hamilton Starlet liquid handling robot coupled to a Cytomat incubator. After another 24 h, the medium was removed and Triton® X-100 lysis buffer (10 µL/well) was added using the BioTek EL406 washer/dispenser. Finally, illuminate mix was added to each well and the light signal was measured in a luminometer (TECAN Infinite Pro) for 0.017 min/well (i.e. ~1 s).

2.2.9.3 Graphs and Statistics

MCF-7 Exposure Studies

Cell counting data were processed as follows; the background (ethanol control) values were subtracted from all raw data before plotting using GraphPad Prism software (version 7.0a for mac, GraphPad Software, La Jolla California, USA, www.graphpad.com). All results are presented as mean ± standard error of n experiments (SEM).

ERα and ERβ CALUX® assays

Luminometer data were processed as follows; the background (DMSO control) values were subtracted from all raw data before plotting using GraphPad Prism software (version 7.0a for mac, GraphPad Software, La Jolla California, USA, www.graphpad.com). A sigmoidal dose-response curve was fitted to the data and EC_{50} values and Hill slopes were determined using non-linear regression analysis.

For the combined exposures, the 'fixed' concentration of the compound present in the background was recalculated to the corresponding concentration of the dose-response-curve compound. This was done using its EC_{50} values as a measure of relative potency. For example, if compound A is a 10x more estrogenic than compound B, 1.0×10^{-9} M of compound A can be recalculated to 1.0×10^{-8} M of compound B. This way, a dose-response curve could be plotted with the total estrogenic potency present in the mixture on the x-axis, expressed as the concentration of the dose-response-curve compound and light emission (or E2 response equivalents) on the y-axis.

In addition, in order to correctly assess the total estrogenicity of the mixtures, the CA model was used (Katchy, *et al.*, 2014). The concentration-addition values were calculated using the equation:

$$R = \left[\frac{1}{1 + \left(\frac{1}{\Sigma(\frac{Ci}{EC_{50}i})^{Average\ hill\ slope}} \right)} \right] \times 100$$

R is the predicted response of the mixture in 'percentage activation' relative to E2, Ci is the concentration of each individual chemical in the mixture, and $EC_{50}i$ is the EC_{50} value of each individual chemical in the mixture. Observed and predicted data for mixture exposures were plotted in GraphPad Prism using a nonlinear regression curve fit.

Cytotox CALUX® assay

Luminometer data were processed as follows; the background (DMSO control) values were subtracted from all raw data before plotting using GraphPad Prism software (version 7.0a for mac, GraphPad Software, La Jolla California, USA, www.graphpad.com). Data was plotted using regression analysis.

2.2.10. Schrödinger Modelling

2.2.10.1. Software Tools

The Schrödinger Suite 2017 is a proprietary software package for studying biomolecular interactions. The various modules and workflows of the suite are accessed through a graphical interface (Maestro). For this work, the applications and workflows listed in Table 2.5 were used. The Schrödinger Suite has been extensively tested and compared to other software tools and its performance is consistently in the top half of the group of tested software tools (Cheng, *et al.*, 2009, Englebienne, *et al.*, 2009, Halgren, *et al.*, 2004, Kellenberger, *et al.*, 2004, Perola, *et al.*, 2004, Repasky, *et al.*, 2007) and often one of the top-ranked tools for docking calculations.

Table 2.5: Schrodinger Suite 2017 applications used in this work.

Application	Task
Maestro (version 11.1.012)	Graphical interface for all Schrodinger applications and workflows
LigPrep	Generation of accurate 3D molecular models for ligand molecules, including tautomeric, stereochemical, and ionisation variation optimised for further computational analyses. These tools are based on well-known bond length, bond angle and atom size parameters and molecular geometry and bonding rules.
Glide	An extensive validated ligand placement algorithm and scoring function package to accurately predict binding mode for ligand-receptor complexes.
Prime	Protein structure predictions, protein structure refinement and MM-GBSA calculations.
Workflow	Task
Protein Preparation Wizard	Correcting common structural problems (e.g. missing residues or residue side chains) and creating reliable, all atom protein models.
Induced Fit Docking	A novel method for fast and accurate prediction of ligand induced conformational changes in receptor active cavities. Combines Glide and Prime to exhaustively consider possible binding modes and the associated conformational changes within the receptor binding cavity.

The process for docking as implemented in the Schrodinger Suite is illustrated in Figure 2.1. The ensemble of poses obtained as output from this process were further evaluated to obtain information from force field scoring functions.

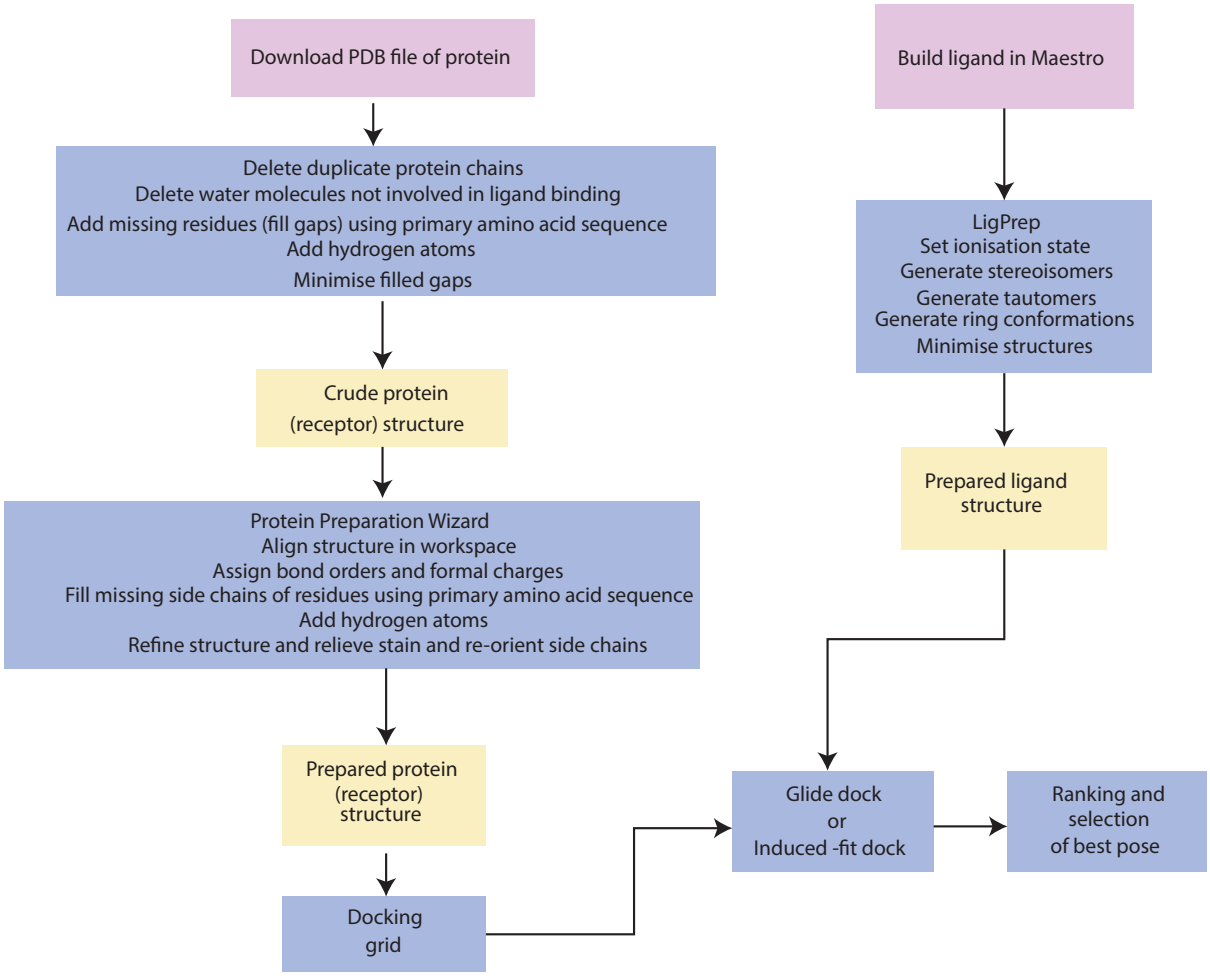


Figure 2.1: Schrodinger Suite ligand docking process.

2.2.10.2. Receptor Model Development

The x-ray crystal structures of the ER LBDs listed in Table 2.2 were used to develop models for docking. Where multiple polypeptide chains were present in the PDB file, chain A was arbitrability selected (because even single polypeptide proteins have chain A) for the experiment. All water molecules except for the one involved in the hydrogen bond bridge with Glu353 and Arg394 in ERα models and Glu305 and Arg346 in ERβ models were removed. Protein Preparation Wizard was used to assign bond orders, add hydrogen atoms and fill in missing side chains within the protein structure. The modified protein structure was then energy minimised to optimise bond distances and angles.

2.2.10.3. Ligand Structure Development

For each ligand, LigPrep was used to determine the ionisation state for carboxylic acids and amines at pH 7 ± 2 (this is close to the cellular pH of 7.4); generate tautomers (e.g. keto-enol); generate alternative chiralities for all stereocentres and sample ring conformations to find lowest (i.e. the most thermodynamically stable) energy conformations. Finally, the geometry of each structure was optimised which involved allowing the structure to relax in all dimensions to achieve a low energy conformation. Not all possible isomers were produced because of internal filtering to eliminate structures which violate geometric restrictions such as for fused ring systems or conflict with natural product chiralities such as for the steroid framework. For some ligands LigPrep produced more than one stereoisomer (e.g. 17α- and 17β-estradiol were produced). For those ligands where it was known which stereoisomer was the desired or correct option, only that stereoisomer was used for docking (e.g. only E2 was used). For equol, 2 possible isomers were produced; however, only the S isomer is found *in vivo*; therefore, the S-isomer was used in the study.

2.2.10.4. Rigid Receptor Docking – RRD

RRD was carried out using Glide and was run within Maestro using the applications menu. The first step in Glide docking was to generate a receptor grid. The LBC binding cavity was defined automatically based on the size, shape and location of the co-crystallised ligand of the particular receptor model used (e.g. 1ERE). For the AF-2 site the centroid of the residues Lys362 and Glu542 in ERα models and Lys314 and Glu493 in ERβ models were used to define the location of the binding cavity. The size was determined by specifying ligands to be docked less than 20 Å in length because it must fit the receptor site. The receptor grid was kept rigid – with no van der Waals radius or change scaling of receptor atoms. The ligand

was docked flexibly, allowing nitrogen inversions and ring conformations. No constraints (e.g. required hydrogen bonds or covalent bonds to metal atoms) were specified and all receptor hydroxyl groups were allowed to rotate.

Glide XP was used for docking using default parameters. The core, constraints and similarity options were not used. The core options allow for constraint of ligand poses within the binding cavity based of a reference ligand. The similarity options incorporate a similarity between a docked ligand and a reference ligand – a measure of how alike or unlike the two molecules are. Both the core and similarity options are useful in virtual screening application for drug target development to aid in the identification of strong binding ligands from a pool of potential candidates, which were not applicable to the task at hand. All the ligands listed in Table 3.3 were docked with receptor models listed in Table 3.2. For RRD, poses were ranked by XPGlideScore which was reported in units of kcal/mol and converted to kJ/mol.

2.2.10.5. Induced-fit Receptor Docking - IFD

IFD was run from within Maestro using the workflow menu (Table 2.6). For the LBC, the Glide grid was defined automatically based on the size and orientation of the co-crystallised ligand. The AF-2 site Glide grid was defined using the centroid of the residues Lys362 and Glu542 in ERα models and Lys314 and Glu493 in ERβ models estimating the location of the binding cavity and the size was determined by specifying ligands to be docked less than 20 Å in length. Initial Glide docking allows residue side chains to be trimmed (temporarily removing them from the protein structure) automatically, based on the B-factor. The B-factor is also called the temperature factor and the higher the value, the more mobile the residue side chain. The more mobile side chains were trimmed as their spatial positions were less certain. The parameters used are summarised in Table 2.6. During the induced fit refinement step, the residues that were allowed to adapt to the placement of the ligand were those within 5 Å of the co-crystallised ligand.

For IFD, poses were ranked by IFDScore. The IFDScore is defined as the sum of XPGlideScore and 5% of the Prime energy (i.e. the total energy of the receptor-ligand complex), so this ranking accounts for the overall energy of the complex, not just a favourable binding energy. The calculated binding energy is given by the scoring function XPGlideScore.

Table 2.6: IFD parameters.

Glide Grid Setup	
Box Centre	Centroid of ligand
Box Size	Automatic based on co-crystallised ligand for LBC docking, centroid of Lys362/314 and Glu542/493 for AF-2 docking
Step 1: Initial Glide docking	
Protein Preparation constrained refinement	Yes
Trim side chains	Yes, automatic based on B-factor
Receptor van der Waals scaling	0.7
Ligand van der Waals scaling	0.5
Number of poses retained per ligand	5
Step 2: Prime induced fit refinement	
Refine residues	Within 5 Å of ligand
Step 3: Glide re-docking	
Glide Docking	XP mode; re-dock into structures within 30 kcal/mol of best structure and within the top 5 structures overall

2.2.10.6. XP GlideScore

XPGlideScore was used in both RRD and IFD to estimate binding energy of the ligand with the receptor. XPGlideScore is optimised to identify and estimate ligand poses that have unfavourable energies based on well-known principles of physical chemistry (e.g. inappropriate hydrophobic interactions, poor hydrogen bonding interactions, etc.) to weed out false positives. As a result, more precise poses are produced than with standard GlideScore; however, the cost for this increased precision is computational time. Standard GlideScore is used for ligand database enrichment to quickly identify good candidates; however, for a more thorough study XPGlideScore was used.

2.2.11. Questionnaire Study

2.2.11.1. Study Design

This was a two-part Canterbury population based cross sectional study. A cross sectional study is a study that assesses disease and exposure in a measured population. In this study, two groups of participants were selected: (1) Women aged between 18-69 years who were randomly selected from the New Zealand Electoral Roll and asked to complete a daily habits questionnaire, and (2) women from the University of Canterbury (UC) staff and students' lists were randomly selected to complete a daily habits questionnaire and donate a blood sample for analysis of xenoestrogens. Additionally, women in both groups were asked to

complete a questionnaire on behalf of their daughters, if they had a daughter who had not had her first period (e.g. menarche), to assess the pre-pubertal exposure to xenoestrogens in girls. If a woman had two daughters who had not had their first period, she was asked to complete the questionnaire for her eldest daughter.

2.2.11.2. Study Area

The study was conducted in Canterbury, a province of Aotearoa-New Zealand which has a land area of 45,346 km^2 and population of 539,436 according to the 2013 Census. The questionnaire was in English which is spoken by most New Zealanders and immigrants.

Undertaking a cross-sectional study in Canterbury was appropriate to get access to participants, and to estimate xenoestrogen exposure, using a random sample of the population (Group 1) and to compare to the findings from the questionnaire with the blood analysis component in Group 2. Also streamlining access to the Canterbury population was relatively easy due to all New Zealand citizens and permanent residents 18 years and above being required to register on the parliamentary electoral roll. UC staff and students' lists were easily accessible and encompassed the desired stratification of age frequencies required for Group 2. Also, of interest for the current study, breast cancer is the most common cancer diagnosed in women in New Zealand and Canterbury, making it highly relevant to our target population.

2.2.11.3. Study Population

The study population consisted of women aged 18-69 years and their pre-pubertal daughters who resided in the Canterbury province. Breast cancer is generally a disease of post-menopausal women, with menopause occurring between the ages of 42-56 years. Our assessment of women across a broad range of ages is important in the context of xenoestrogen mimic exposure and its potential breast cancer risk. Although women below the age of menopause (mean 51.5 years) are less likely to have developed breast cancer, their food and lifestyle habits now could be influencing their overall breast cancer risk later in life. By the age of 69 years the number of potential participants in the population significantly decreases; therefore, women over 69 years were not sought for the study. Additionally, women were asked to fill in an additional questionnaire on behalf of their daughter, only if they had one, who had not reached menarche (i.e. had not had her first period).

2.2.11.4. Inclusion and Exclusion Criteria

Women in Group 1 were randomly selected from the General and Māori Electoral Rolls for the Canterbury province using a statistical analysis system (SAS) random number generator. All males were removed from the lists. Women whose name was not on the electoral rolls or who were 70 years and older were excluded from the study. All participants gave informed consent to participate in the study and, if applicable, gave consent on behalf of their pre-pubertal daughter. Women in Group 2 between the ages of 18-69 were selected from UC staff and student lists. All women were capable of completing a self-administered questionnaire, gave consent to participate in the study and gave consent on behalf of their daughter if appropriate. Participants in this group were also capable of donating a 20 mL blood sample.

2.2.11.5. Sampling

There was sequential recruitment of participants until the target of each of the studies was achieved. The participants for the questionnaire study (Group 1) were selected randomly from the electoral rolls. Each person who met the inclusion criteria (see Section 2.2.11.4.) was assigned a random number between 0 and 1. They were then assigned a unique ID that incorporated the random number and their age (as at midnight on the 21st of September 2016). This was then used to select participants in the correct frequency matched 5-year age groups. Participants in the blood analysis study were sequentially recruited through the use of UC staff and student email lists until the desired sample size was achieved in the correct frequency matched 5-year age groups. Samples of the letters to both groups of women are provided as Appendix 6.

2.2.11.6. Sample Size

When deciding the sample size, it was important to consider the number of study participants required to answer the study objectives. A small study may fail to detect important effects on the outcome of interest, whereas a study larger than necessary wastes resources. Furthermore, the study must be appropriate to be completed in the time frame of this PhD. Taking account of these considerations the first group target was 250 participants and the second group were 50 participants. The following equation has been used to estimate sample sizes for surveys (Schaeffer *et al*., 1990) and was used to estimate an appropriate sample size (n) for the questionnaire study (Group 1):

$$n = deff\frac{Npq}{\frac{d^2}{1.96^2}(N-1)+pq}$$

N is the population size, *p* is the estimated proportion, *q* is 1-*p*, *d* is the desired absolute precision, and *deff* is the design effect. The value of 1.96 is based on the fact that 95% of the area of a normal distribution is within 1.96 standard deviations of the mean.

At the start of the study there were limited data available on the prevalence of xenoestrogen exposures in New Zealand; however, a 2005 study (Thomson, 2005), which investigated dietary xenoestrogen exposures, suggests people are likely exposed to a number of dietary xenoestrogens on a daily basis. Therefore, taking this into consideration with other possible xenoestrogen exposures (e.g. parabens), a conservative hypothesised frequency of 80% was selected. The estimated population size of women in Canterbury is 184, 700 (based on 2013 NZ Census data) and the design effect was set to one as this is a random simple sample. Using this data, it was determined that a sample size of 250 would provide statistically significant sample with 95% confidence intervals.

The blood analysis study was not intended to be statistically robust but rather to give an indication of xenoestrogens present in women's blood. It was decided to set up a study with the University of Canterbury Medical Centre to take blood samples from 50 women who were attending the medical centre for other reasons and agreed to take part in the study or had responded to a UC staff and student email. It was decided to include 50 women in this study because that was considered appropriate number to sample in a working day. The number was not statistically driven as this was a pilot study.

2.2.11.7. Data Collection Instrument

The data were collected using a structured self-administered questionnaire (Appendix 6) covering the following areas:

1. Socio-demographic characteristics of the study participants: these included age, occupation, area of residence and weight. These were to help judge whether the selected group represented the population by comparing to other studies including the New Zealand Dietary survey. Additionally, their weight was asked to help us calculate the average amount of blood for a person in that particular age group.
2. History of breast cancer: this covered their personal experience to having breast cancer and what subtype of breast cancer they had (e.g. ER+ve, ER-ve etc.). This provided insight into their food and lifestyle habits and allowed adjustment for possible changes in diet related to a previous/current breast cancer diagnosis.

3. Hormone medication: this covered current usage of contraceptives, hormone replacement treatment and/or fertility treatments. By taking any of these medications, the overall estrogenic load experienced by participants is significantly increased and may make other exposures negligible.
4. Food and lifestyle habits: this included questions on food consumption, food packaging use, personal care product usage, dietary supplementation regimes and other non-hormonal medication usages. These covered all the main routes of xenoestrogen exposure.
5. Elimination of xenoestrogens: this covered whether the participant had heard of xenoestrogens prior to the questionnaire and whether they had taken any steps to eliminate them from their daily routine.

The questionnaire included questions derived from the NZ Census and questions seeking further information sourced from suitable questionnaires including those used in previous dietary surveys. The use of questions based on information provided by the NZ Census allowed for comparison to the source population. The questionnaire was pilot tested and revised before being used in the study. The participants were also given the option of receiving a report of the study results but not a report of their individual results.

2.2.11.8. Data Collection Procedure

Recruitment of study participants and data collection was done Canterbury wide. All women were taken from both the General and Māori Electoral Rolls and placed into a spreadsheet in age order as of the 21st September 2016. Each woman was assigned a random number using a SAS random number generator. The women were then ordered numerically according to their randomly assigned number. The first 750 women were selected for recruitment to the study. The selected women were sent an information sheet detailing the study, a consent form, a copy of the food and lifestyle habits questionnaire and a copy of the daughter's questionnaire and a postage paid addressed envelope for returning the questionnaire/s and consent form (Appendix 6) (Fig. 2.2).

Access to electronic data about people on the New Zealand Electoral roll for purposes of health research is allowed under section 112 (3) of the (NZ) Electoral Act 1993. To facilitate responses, women who did not respond to the initial questionnaire and consent form within 3 months from the dispatch date, were sent a second questionnaire package. The second round of dispatches also included an Ethics Committee amendment, which allowed an online option

for questionnaire completion. Those selected were informed that by pressing the submit button, they were consenting to the information provided to be used in the study. Those participants who returned completed questionnaires without a signed consent form were considered to have consented.

The recruitment of study participants and data collection for Group 2 was done within the UC staff and student lists. Women were informed of the study and asked to participate in the study via email. Participants were asked to come to the University of Canterbury Health Centre on specified days and the first five willing participants of each age frequency were selected to participate in the study (Fig. 2.2).

Figure 2.2: Questionnaire and blood analysis data collection processes

2.2.11.9. Ethical Considerations

Ethics committee approval was received for this part of the research from the University of Canterbury Human Ethics Committee (Approval number 2016/45). The approval letter (Appendix 7) and information sheets, consent forms and questionnaire are included in Appendix 6. In addition, the study proposal was assessed by the University of Canterbury Māori Research Advisory Group.

2.2.11.10. Data Management and Analysis

Questionnaire

Questionnaire data (both groups) were entered into an excel spreadsheet. Frequency tables were then constructed in order to aid in identifying data entry errors. These errors were corrected by referring to the answers in the questionnaires. All identifying information was coded before analysis.

Data were analysed using the IBM Statistical Package for the Social Sciences (IBM SPSS statistics). Descriptive statistics were used to compute frequencies in each of the five-age frequencies.

Blood Xenostrogen Concentration Data

New Zealand data (see Appendix 5) were used to estimate the xenoestrogen concentrations in foods and personal care products whenever they were available for population consistency with consumption and application data. Where New Zealand data were not available, data from the world scientific literature were employed. Attempts were made to use data from populations that are as culturally similar (e.g. Australia) to New Zealand as possible.

Quantifying Food Servings and Personal Care Product Applications

Food serving information from the 2009 New Zealand Total Diet Survey (Vannoort*, et al.*, 2009) was used to determine serving sizes for a simulated New Zealand typical diet for specified subgroups (Section 7.2.1.). However, the number of foods included in the New Zealand Total Diet Survey limits the usefulness because it does not include every possible food that is consumed by New Zealanders. Therefore, if a food was not included in the New Zealand Total Diet Survey the serving size for a similar food was used to estimate the serving size. For example, parsnip was not included in the 2009 New Zealand Total Diet Survey, but carrots were; therefore, based on the similarity in serving size and the fact that carrots and parsnips are both root vegetables, it was assumed the serving size for parsnip was similar as

for carrots. In addition, personal care product application size (Appendix 5) were taken from world scientific literature and applied to the xenoestrogen exposure assessment. Attempts were made to use data from populations that are culturally similar to New Zealand as possible.

Relative Estrogenic Potency Data

Relative estrogenicity (in estrogen equivalents (EQ)) was based on *in vitro* competitive binding or gene expression assays because they assess the basic binding to ERs without the higher level of biological complexity (e.g. xenobiotic metabolism) often associated with cell proliferation assays, such as the MCF-7 proliferation assay. EQ is the concentration of E2 required to achieve 50% of the maximal response (EC_{50}) divided by the concentration of the test compound required to achieve the same effect. EQ estimates are widely used to characterise and compare the potency of a wide variety of compounds, in this case xenoestrogens. For internal consistency, results from ERα and ERβ CALUX® experiments in Chapter 5 were also used. Using EQ, the total daily estrogenic load for each subgroup was calculated. This was carried out by calculating the mass (e.g. dose) of the food or personal care product, how much was eaten or used (e.g. serving or application size) and the concentrations of xenoestrogens in the food or personal care product, and therefore how much xenoestrogen participants were exposed to relating to their consumption/product usage. EQs were then determined for the calculated xenoestrogen concentrations (X) from literature EC_{50} values as follows:

$$\mathrm{EQ} = \frac{EC_{50}\ [E2]}{EC_{50}\ [X]}$$

Circulating Concentrations of Xenoestrogens

The average total blood volume for each subgroup of women was calculated using the following formula (Nadler*, et al.*, 1962):

$$Blood\ Volume = Body\ Weight\ (kg) \times\ Average\ Blood\ Volume(\frac{mL}{kg})$$

The average blood volume/body weight (mg/kg), where the density of blood was assumed to be 1 mg/mL (Vitello*, et al.*, 2015), was based on published literature values (Morgan*, et al.*, 2002): namely, 65 mg/kg for women and 80 mg/kg for pre-pubertal girls. In the questionnaire, women were asked to report their weight from which an average weight was calculated for each subgroup group.

Once the total blood volume was calculated, the total concentration of xenoestrogens (as EQ)/L blood was calculated for each subgroup of women and pre-pubertal girls. The daily xenoestrogen intake had been calculated previously. This was assumed to be equally distributed in the circulatory system. Therefore, the circulating xenoestrogen concentration was determined as follows:

$$\frac{Calculated\ total\ xenoestrogen\ exposure}{Calcuated\ total\ blood\ volume}$$

Circulating E2 Concentrations

The estimated concentration of circulating E2 were determined from the scientific literature for each subgroup of women and pre-pubertal girls (Table 2.7).

Table 2.7: Circulating E2 concentrations

Subgroup (age range)	E2 concentration range (M)
Women	
18-19	$9.9 \times 10^{-11} – 1.6 \times 10^{-9}$
30-39	$9.9 \times 10^{-11} – 1.6 \times 10^{-9}$
40-49	$9.9 \times 10^{-11} – 1.6 \times 10^{-9}$
50-59	$0.0 – 1.5 \times 10^{-10}$
60-69	$0.0 – 3.7 \times 10^{-11}$
Pre-pubertal girls	
0-12 months	$0.0 – 1.5 \times 10^{-11}$
1-3	$0.0 – 1.5 \times 10^{-11}$
5-6	$0.0 – 1.5 \times 10^{-11}$
11-14	$0.0 – 1.3 \times 10^{-9}$

The total concentration of E2 was determined, the total estrogenic loads for each subgroup of women and pre-pubertal girls were calculated. It was assumed that E2 and the xenoestrogens were equally distributed in the circulatory system. The total estrogenic loads were calculated by adding the calculated xenoestrogen concentration in EQ (M) to the determined E2 (M) concentration.

2.2.12. Blood Analysis

2.2.12.1. Sample Collection

Blood samples (20 mL) were collected using a vacutainer and needle from each of the participants in Group 2. The samples were left for a minimum of 30 min at room temperature to clot before being stored at 4°C.

2.2.12.2. Serum Preparation

Blood samples were processed in batches of 12. The samples were centrifuged (2000 xg for 10 min), on the same day they were collected. The serum layer was removed using a Pasteur pipette, transferred into a glass scintillation vial (20 mL) and stored at -20°C until analysed.

2.2.12.3. Serum Extraction

The serum samples were thawed at room temperature. Aliquots (10 mL) of serum were extracted using methyl *tert*-butyl ether (10 mL). The serum/ether mix was inverted 10 times and allowed to settle. The ether (upper) layer was transferred using a glass Pasteur pipette into a clean test tube (20 mL) containing anhydrous sodium sulfate (approx. 10 g). The

sodium sulfate was used to remove any remaining water from the samples. The solution was filtered using whatman filter paper (grade 2, 8µm) and transferred to a clean scintillation vial (20 mL). The samples were dried using a vacuum concentrator (SpeedVac SPD121P, Thermofisher) at 37°C for 20 min. The extracts were stored at 4°C overnight. The sample extracts were dissolved in analytical grade acetonitrile. If the samples did not fully dissolve a few drops of chloroform were added. The dissolved extracts were transferred to HPLC vials and analysed for the target analytes using LC (Dionex Ultimate 3000, ThermoFisher) coupled with a MS (maXis 3G UHR-Qq-TOF, Bruker Daltonik GmbH, Bremen, Germany).

2.2.12.4. Serum Analysis

For detection of all analytes, each sample was analysed using LC-MS coupled with the Bruker Compass Data Analysis software program (Bruker Daltonik GmbH, Bremen, Germany). The LC-MS system was equipped with an electrospray ion source (ESI) and was running in positive mode. The sample (5 µL) was injected onto a C18 reverse phase HPLC column (Dionex). The flow rate was 200 µL/min, and the column temperature was 25°C. Solvents were A: 0.1% v/v (aq.) acetic acid and B: 0.1% acetic acid in acetonitrile. Table 2.8 shows the solvent programming. An analyte was positively detected if the mass ion was identified within 2 decimal places of the analyte standard's accurate molar mass and had the same retention time.

Table 2.8: Solvent gradient: A, 0.1% acetic acid in water; B, 0.1% acetic acid in acetonitrile

Time (min)	0.0	1.5	1.6	6.0	6.1	10.0	10.1	12.0	15.5	15.6	17.0
Solvent A (%)	95	95	73	70	55	55	30	10	10	95	95
Solvent B (%)	5	5	27	30	45	45	70	90	90	5	5

Chapter 3 Understanding the MCF-7 Model System

3.1 Introduction

MCF-7 cells are the most studied human breast cancer cell line in the world (Lee, *et al.*, 2015). Results from these studies have made a fundamental impact upon breast cancer research, particularly for ERα positive breast cancer. To date there have been nearly 25,000 published reports on this cell line, rivalled only by the nearly 80,000 publications using the HeLa cell line (Lee, *et al.*, 2015). The popularity of the MCF-7 cell line for breast cancer research reflects its reliability to many aspects of breast cancer in a clinical setting, particularly in the understanding of postmenopausal women with hormone receptor positive breast cancer.

3.1.1. A Brief History of MCF-7 Cells

The MCF-7 cell line was established in 1973 by Dr. Soule and colleagues at the Michigan Cancer Foundation (Soule, *et al.*, 1973). MCF-7 cells, named after the Michigan Cancer Foundation and representing Dr. Soules seventh attempt at generating a cancer cell line, were isolated from the pleural effusion of a 69 year old women with metastatic breast cancer disease. The patient had undergone a conservative radical mastectomy for malignant breast adenocarcinoma three years earlier. Local recurrences were controlled for the subsequent three years with radiotherapy and an unrecorded hormone therapy, most likely the synthetic estrogen diethylstilbestrol. Two months after widespread nodular recurrences, cells were excised from chest wall nodules and from a pleural effusion. The chest wall cells quickly became overgrown in culture by fibroblasts; however, the pleural effusion cells initially grew in suspension and then ultimately formed a monolayer on plastic culture flasks that grew in continuous culture, a technique where cells are maintained in a particular phase of growth (e.g. log phase; Fig. 3.1). At this time cell culture was still in its infancy, with the first cancer cell line (HeLa) being established some 20 years earlier. Many laboratories documented technical difficulties in generating homogenous breast cancer cell populations without significant stromal contamination and overgrowth by fibroblasts. Several laboratories had

tried to isolate cells using different substrates and nutrients with many cell lines only capable of being cultured for a few months at a time (Levenson*, et al.*, 1997, Soule*, et al.*, 1973).

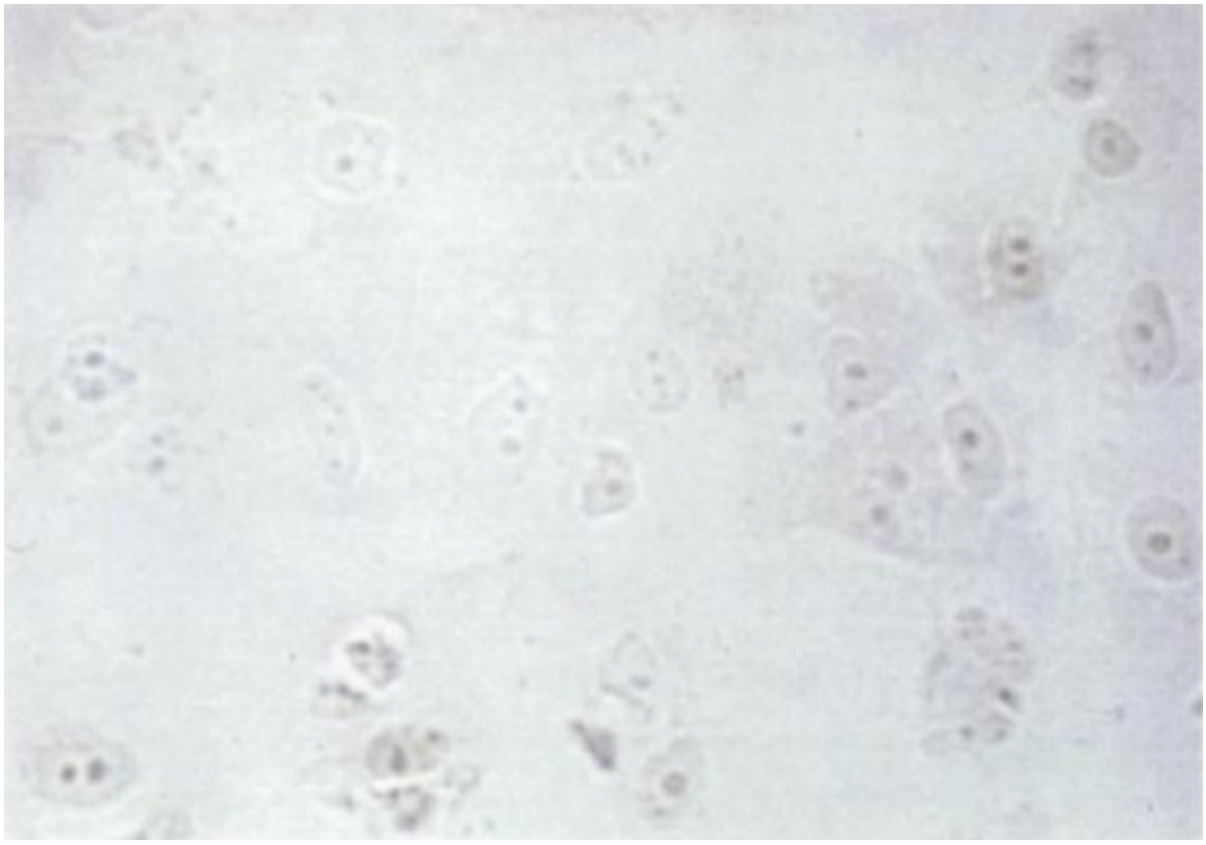


Figure 3.1: Historical image of MCF-7 cells (image from Levenson and Jordan 1997 with permission).

3.1.2. MCF-7 and the ERs

The MCF-7 cell line has made important contributions to both breast cancer research and our current understanding of the ERs. This cell line is one of very few established breast cancer cell lines able to express substantial levels of ERα, akin to a majority of invasive breast cancers. The maintenance of ER expression in cultured cells lines is difficult and has resulted in far more ER-ve than ER+ve human breast cancer cell lines to date. Evidence of this loss in ER expression in cultured breast cancer cells points towards the role of extracellular matrix and inappropriate growth substrate (i.e. two-dimensional plastic). Interestingly, it appears Dr. Soule did not perform any modifications to the standard isolation technique at the time to specifically isolate an ER+ve cell line. In the original description of MCF-7 cells, Dr. Soule did not make reference to the ERα +ve expression. This finding was subsequently described in another publication in 1973 where Soule and his colleagues reported the finding of "specific estrogen receptor in MCF-7…" (Brooks*, et al.*, 1973). The importance of ER

expression and how this would impact the basic understanding of ERs at the time was also recognised, with the authors acknowledging the potential to "add to present knowledge regarding intracellular binding constants, transport mechanisms and the mode of nuclear uptake". However, it was not until 1975 when Lippman (Lippman*, et al.*, 1975) and Horwitz (Horwitz*, et al.*, 1975) first reported the ER status and biological function of MCF-7 cells, with both clearly realising and stating the importance the cell line would have in breast cancer research in the future.

3.1.3. Early MCF-7 Studies

While it appeared straightforward to use the MCF-7 cell line to understand the actions of the ER, the simple demonstration of stimulation of MCF-7 cell growth by E2 was more challenging and not as reproducible as anticipated (Osborne*, et al.*, 1983, Sutherland*, et al.*, 1983). In 1986, the Katzenellenbogens discovered that phenol red, used in tissue culture media as an indicator of pH, was a weak estrogen and was sufficient to induce activation of the ER at the high concentration used in media. The removal of phenol red eliminated the confounding variable and was a critical step forward in understanding how estrogen activated the ER and stimulated breast cancer cell growth (Berthois*, et al.*, 1986). Solving this predicament was absolutely critical for future research into ER action in MCF-7 cells. Comparatively, early studies on anti-estrogens were much more straight forward with inhibition being reported of the MCF-7 cell cycle at the G0/G1 block aid, leading to cell proliferation inhibition (Osborne*, et al.*, 1983, Sutherland*, et al.*, 1983).

In addition, MCF-7 cells were central to the development of ER antibodies with the first monoclonal antibody for human ER being purified from this cell line (Greene*, et al.*, 1980).The antibodies aided in the cloning and sequencing of ESR1(Walter*, et al.*, 1985) and the clarification of prominent nuclear localisation of ER (King*, et al.*, 1984) thus providing key insights into the ER mechanism of action upon E2 activation.

It is also important to note that while MCF-7s are viewed as the "work horse" for studies of estrogen action in breast cancer, these cells also express androgen, progesterone and glucocorticoid receptors (Horwitz*, et al.*, 1975). MCF-7 cells have served as a valuable model system to elucidate other pathways of hormone response and resistance.

3.1.4. Genomics of MCF-7 Cells

Understanding the variability and isolation of MCF-7 is critical, as fundamental concepts of breast cancer have been developed from this single cell line. In the original report on the isolation of MCF-7, chromosome number was assessed in passage two of the pleural effusion and found to range from 70-144 (Soule*, et al.*, 1973). However, analysis of the MCF-7 cell line at passage 39 showed that the chromosome number had narrowed to a range of 77-99. Remarkably, this number has stabilised further over the last 45 years with the current number of chromosomes in MCF-7 cells provided by the American Type Culture Collection (ATCC) is 82 (range 66 to 87) (Lee*, et al.*, 2015).

When considering the increased understanding of breast cancer cell heterogeneity and evolution it is not surprising that there is a considerable amount of genomic instability. Some studies have also shown different gene expression and genomic profiles, while others have shown karyotype differences between MCF-7 cell line variants (Osborne*, et al.*, 1987). Indeed, this genomic instability is akin to breast cancer in a clinical setting where, in a patient over time, the breast cancer cells exhibit similar genomic instability, whether that be from therapies (e.g. hormonal treatment) or spontaneously.

One of the most disconcerting aspects of MCF-7 cells in the laboratory has been their ability to adapt and evolve over time, such that many different variants of the original cell line have been identified. These adaptations have led difficulty to replicate results from different MCF-7 variants from different laboratories around the world (Lorsch*, et al.*, 2014). Initially, cross-contamination was thought to be the general issue, like with many other cell lines; however, it is now thought that genomic instability of the cell line is responsible for the variation between MCF-7 culture variants. It was Osborne and colleagues who first reported the American type culture collection (ATCC) MCF-7 cell line to be cytogenetically dissimilar to the original MCF-7 cell line described by Dr. Soule (Osborne*, et al.*, 1987). Since then investigators from other laboratories have also identified this issue and have even noted different responses between MCF-7 cell line variants in biologic assays. Many laboratories now distinguish their MCF-7 cell line by naming their own variants including MCF-7 L (Lippman, B (Benz), KP (Kent Osborne), BK (Benita Katzenellenbogen), and many more. Therefore, with the likelihood of many different MCF-7 cell line variants around the world, it is obvious many different adaptations have been developed for culture media formulation for culturing MCF-7 cells.

3.1.5. The MCF-7 Culture System

Culture medium is the most important factor in cell culture technology, it supports cell survival and proliferation, as well as cellular functions; obviously this means that the quality of the medium directly affects research results. An array of different culture media and a diverse range of nutrient formulations have been established for culturing MCF-7 cells over the last 45 years. A commonly used culture medium formulation, as described by Comsa and colleagues, includes low glucose DMEM containing 10% FBS, 2 mM glutamine, 0.01 mg/mL insulin and 1% penicillin/streptomycin mix and incubated at 37ºC in an atmosphere of 5% CO_2 v/v in air (Comsa*, et al.*, 2015). The addition of 1% non-essential amino acid supplementation is also common in culture media used to culture MCF-7 cells (Adams, 1990).

Cell culture has developed in leaps and bounds, with many breakthroughs leading to cell culture equipment being easily accessible to any scientist wanting to work with cultured cells. Despite the clear limitations, the value of MCF-7 cells to the understanding of ER action cannot be overstated. The pivotal work using this cell line continues unabated and when used in the right way remains a powerful experimental tool and continue to lead to improved outcomes in breast cancer patients (Lee*, et al.*, 2015). However, the influence of the culture system on MCF-7 proliferation has not been fully understood.

The aim of this chapter was to understand the culture system that optimised MCF-7 cell growth in a laboratory setting and aligned with international literature with minimal background variability which could influence the results presented in Chapter 4. Additionally, the impact of two trypsin enzyme solutions used during cell maintenance and experimental procedures was investigated to reduce any influence on cell variability and reproducibility.

3.2. Experimental Approach

Since the MCF-7 cell line is one of the most used cells in laboratories around the world, there are plethora of different culture media available for MCF-7 cell culture. For example, culture media with different nutrient supplements (e.g. glutamine), the inclusion of NEAA (e.g. glycine, serine), and key energy sources (e.g. glucose). However, the scientific literature gives little guidance on which medium to use for a particular application – published studies using MCF-7 cells rarely explain their choice in culture medium. For this reason, I embarked

upon an investigation on the most appropriate media for the model system I wanted to develop (see Chapter 4).

Cell culture media composition used in MCF-7 cell culture was evaluated and compared to determine if there were any significant differences between the compositions. MCF-7 cells were then challenged with the different culture media and proliferation was measured using cell counting techniques as previously described (Section 2.2.4.). Three different culture media were tested: MEM, DMEM and RPMI-1640.

Furthermore, the metabolic pathways in MCF-7 cells were investigated with different culture medium supplementations. It is well known that metabolic reprogramming in cancer cells is required to drive biosynthetic pathways which enables rapid cell proliferation. Therefore, two of the most used substrates, glucose (metabolised by glycolysis) and pyruvate (a carbon source for the tricarboxcyclic acid (TCA) cycle) were investigated to evaluate the effect of this category of supplements on MCF-7 cell proliferation.

Trypsin is a serine protease which cleaves two amino acids, arginine and lysine, on cell surface proteins (i.e. cadherin) thus, interferring with cell-cell and cell-matrix (e.g. plastic culture flask) interactions. Treatment with trypsin allows the cells to dissociate from the plastic culture flask and each other to achieve a single cell suspension which is important in reducing variability during dose response and growth curve experiments (see Chapter 4). Two main forms of trypsin are available to use: TrypLE™ Express, a recombinant fungal enzyme produced by fermentation, and porcine trypsin isolated from the pancreas of pigs. The difference between the two types of trypsin is that TrypLE™ Express is described by its manufacturers to be ‘gentler’ at cleaving the cell surface proteins in addition to its increased stability and storage longevity compared to porcine isolated trypsin. Therefore, TrypLE™ Express and a 2.5% trypsin protease solution were tested to determine which trypsin was most effective in the dissociation of MCF-7 cells without affecting cell growth. If either of the trypsin solutions were to affect MCF-7 cell growth prior to an experiment, it could mask any differences observed in subsequent chapters. MCF-7 cells were exposed for 5, 10 and 30 min to TrypLE™ Express (1x). Cells were also exposed to a 2.5% trypsin protease solution and monitored under an inverted microscope. Since the trypsin protease was being used, they were only observed for 5 min to prevent cell death.

The MCF-7 culture system used in this thesis was developed on a modification of the previous culture method used in the Human Toxicology Research Group (Webber, 2013), the general culture media composition described above (Section 5.1.3.) and the method used at the Christchurch District Health Board (CDHB) where the cell line originated. The culture media compositions selected for testing are shown in Table 3.1.

Table 3.1: Culture media formulations (1) (2) and (3) indicate the culture conditions used. The tick indicates the presence of the nutrients in each of the different culture media formulations.

Culture medium	FBS (10%)	Strep/Pen 1%	L-Glutamine	Sodium pyruvate	High glucose (0.025 M)	NEAAs
MEM (1)	✓	✓	✓			
DMEM	✓	✓	✓		✓	
DMEM (2)	✓	✓	✓			
RPMI-1640	✓	✓	✓			✓
RPMI-1640 (3)	✓	✓	✓	✓		✓

3.3. Results

3.3.1. Effect of Culture Medium on MCF-7 Cell Proliferation

To understand the effects of the culture media on MCF-7 cell proliferation, the cells were passaged into each of the culture media formulations described in Table 3.1. For test (1) the cells proliferated slowly (e.g. the cells did not reach confluence until day 10) and presented with a low level of adherence to the culture flask. This is uncharacteristic of MCF-7 cells as they are known to be an adherent cell line and have previously been shown to reach confluence at day 6 (Webber, 2013); therefore, no further experiments were carried out using this culture medium. The next test (2) increased proliferation compared to test (1). However, cells did not rapidly proliferate as expected and exhibited a low level of adherence. Therefore, this culture media formulation was not routinely used, however, it was used in a later experiment to investigate the impact of high and low glucose supplementation on MCF-7 cell growth (see Section 2.2.6.). Finally, test (3) was carried out and the cells reached confluence as expected with a high level of adherence. The MCF-7 cells exhibited an increase in proliferation compared to proliferation observed with tests (1) and (2). The growth curve experiment carried out using this culture method also produced a specific (i.e. middle of log phase reached at day 5) sigmoidal growth pattern characteristic of MCF-7 cells (see Fig. 3.2).

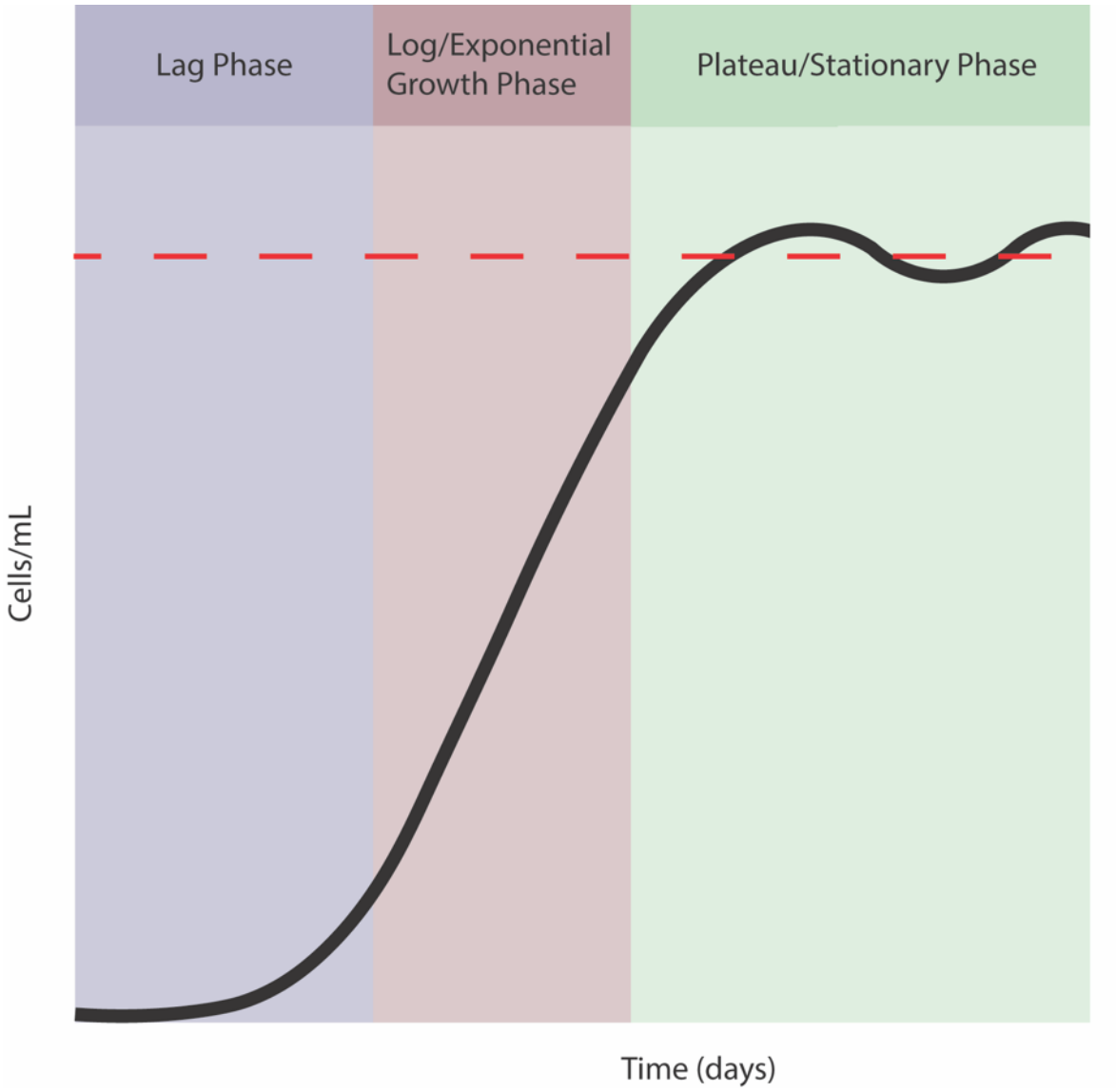


Figure 3.2: A schematic sigmoidal growth curve in culture based on how one would expect cells to grow. The cells would be expected to exhibit a typical three phase growth pattern: 1) the lag phase – cell population grows slowly; 2) the log or exponential growth phase – the cell population undergoes a rapid period of growth 3) the plateau or stationary phase – cell population growth becomes static and cell proliferation = cell death, this is referred to as the carrying capacity (from Bioninja with permission).

Furthermore, when comparing the culture media compositions (Table 3.2) it is clear that there is a high degree of composition commonality between MEM and DMEM. However, RPMI-1640 included non-essential amino acids (L-asparagine, L-aspartic acid, L-glutamic acid, L-proline and hydroxyproline) which were not part of MEM and DMEM's composition. The DMEM culture medium had additional supplementation, including L-serine, glycine, ferric nitrate and pyridoxine hydrochloride, compared to MEM; however, it was unclear whether these compounds affected cell proliferation with both experiments displaying low cell

adhesion to the culture flask in the presence of both culture media formulations. Interestingly, when comparing the culture media compositions with the non-essential amino acids solution available to add to MEM and DMEM culture media, it is clear that the amino acid differences between MEM/DMEM and RPMI-1640 are the same as those included in the NEAAs solution (Table 3.2).

Table 3.2: Comparison of culture media composition.

Component	MEM	DMEM	RPMI-1640	NEAAs
Amino Acids				
Glycine		✓	✓	✓
L-Arginine hydrochloride	✓	✓		
L-Cysteine 2HCl	✓	✓	✓	
L-Glutamine	✓	✓	✓	
L-Histidine hydrochloride-H_2O	✓	✓		
L-Isoleucine	✓	✓	✓	
L-Leucine	✓	✓	✓	
L-Lysine hydrochloride	✓	✓	✓	
L-Methionine	✓	✓	✓	
L-Phenylalanine	✓	✓	✓	
L-Serine		✓	✓	✓
L-Threonine	✓	✓	✓	
L-Tryptophan	✓	✓	✓	
L-Tyrosine disodium salt dihydrate	✓	✓	✓	
L-Valine	✓	✓	✓	
L-Arginine			✓	
L-Asparagine			✓	✓
L-Aspartic Acid			✓	✓
L-Glutamic Acid			✓	✓
L-Histidine			✓	
L-Hydroxyproline			✓	
L-Proline			✓	✓
L-Alanine				✓
Vitamins				
Choline chloride	✓	✓	✓	
D-Calcium pantothenate	✓	✓	✓	
Folic Acid	✓	✓	✓	
Niacinamide	✓	✓	✓	
Pyridoxine hydrochloride		✓	✓	
Riboflavin	✓	✓	✓	
Thiamine hydrochloride	✓	✓	✓	
i-Inositol	✓	✓	✓	

Pyridoxal hydrochloride	✓		
Biotin			✓
4-Para aminobenzoic acid			✓
Vitamin B12			✓
Inorganic Salts			
Calcium chloride	✓	✓	
Ferric nitrate		✓	
Magnesium sulfate	✓	✓	✓
Potassium chloride	✓	✓	✓
Sodium bicarbonate	✓	✓	✓
Sodium chloride	✓	✓	✓
Sodium phosphate monobasic	✓	✓	
Calcium nitrate			✓
Sodium phosphate dibasic anhydrous			✓
Other Components			
D-glucose	✓	✓	✓
Phenol red	✓	✓	✓
Glutathione			✓

3.3.2. Effect of Sodium Pyruvate and Glucose Supplementations on MCF-7 Cell Proliferation

To gain further insights into the influence of culture media supplementation on MCF-7 cell proliferation, MCF-7 cells were grown in DMEM with a high glucose supplementation, and RPMI-1640 with sodium pyruvate supplementation. All culture media used in these experiments contained low glucose (Table 3.2), therefore it was not possible to do a low glucose experiment. When comparing MCF-7 cell proliferation between the groups, no difference was found between a high glucose supplemented culture medium and a low glucose supplemented culture medium. However, the was a significant increase in MCF-7 cell proliferation when sodium pyruvate was present in the culture medium.

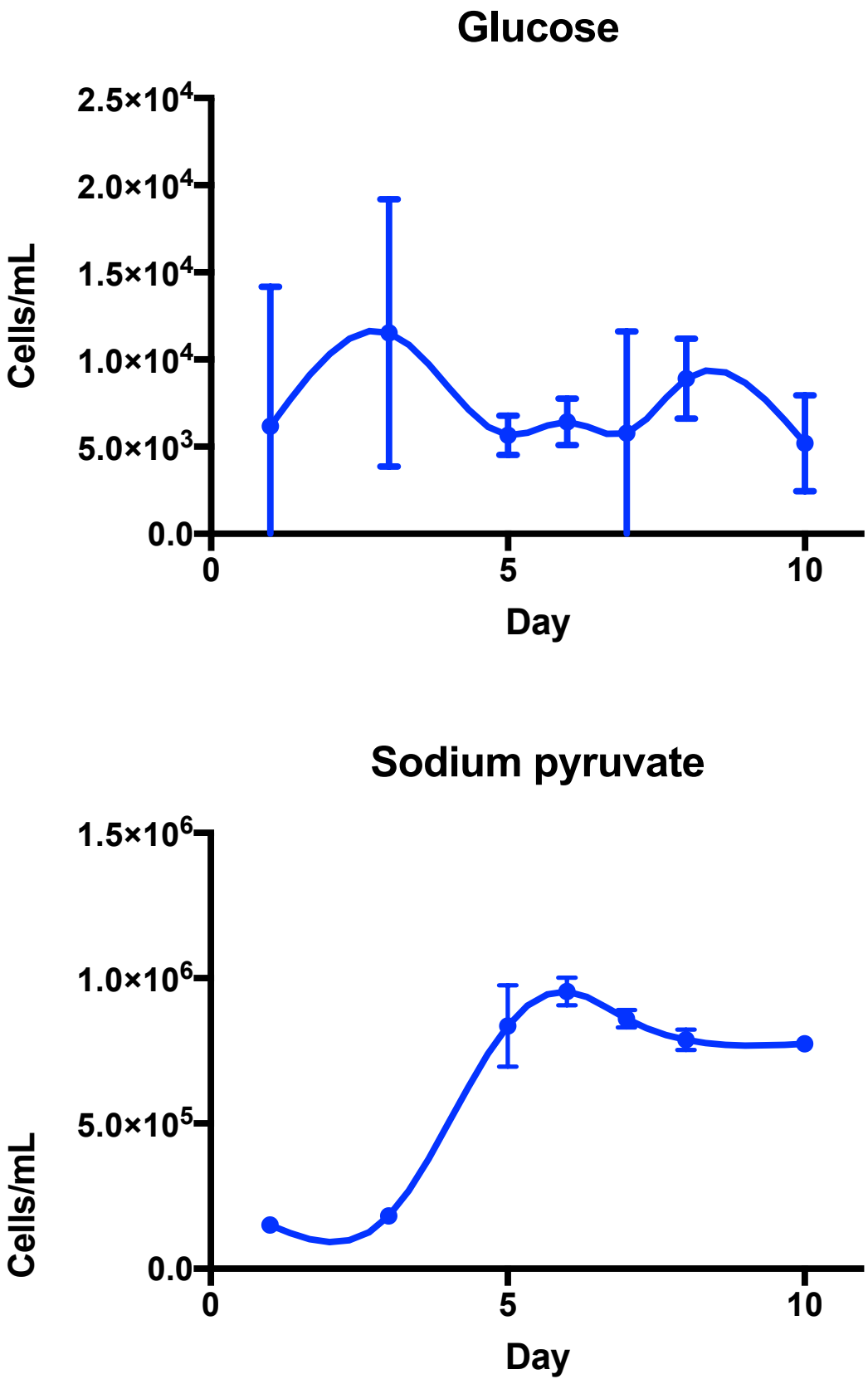


Figure 3.3: Proliferation (± SEM, n=3) of MCF-7 cells exposed to high glucose (a) and sodium pyruvate (b) – containing culture medium.

3.3.3. TrypLE™ Express and Trypsin Protease Dispersal of MCF-7 Cells

MCF-7 cells were exposed to TrypLE™ Express (2 mL) for 5, 10 or 30 min. Cells exposed to TrypLE™ Express for 5 min began to detach, with a majority of the cells remaining adhered to the bottom of the culture flask. However, when the cells were exposed to TrypLE™ Express for 10 min the cells were detached enough to the point where when the culture medium was added, the force from the expelled liquid using the automatic pipette was enough to fully remove the cells from the bottom of the flask. However, when the cells were seeded into 24 well plates to begin an experiment, the cells still adhered to one another (clumped), making an even dispersal almost impossible. This led to a high cell count variability. However, for cell maintenance, complete cell-cell dissociation was not necessary; therefore, an approximate 10 min exposure to TrypLE™ Express was sufficient unless preparing for an experiment where a single cell suspension was required to get an even distribution of cells in each of the wells. If the cell-cell dissociation was unsuccessful the results often had a high variability between replicates. When cells were exposed to TrypLE™ Express for 30 min the cells completely detached from the bottom of the flask; however, the cells dissociated in large clumps which were near impossible to separate without causing damage to the cells. If the cells became damaged they would not adhere to the flask, instead the cells would float in suspension. Eventually this led to cells growing in large floating clumps rather than a monolayer on the bottom of the flask. In addition, cells exposed to TrypLE™ Express for 30 min spent a longer time in the lag phase, resulting in the log phase being delayed for up to three days (Fig. 3.4).

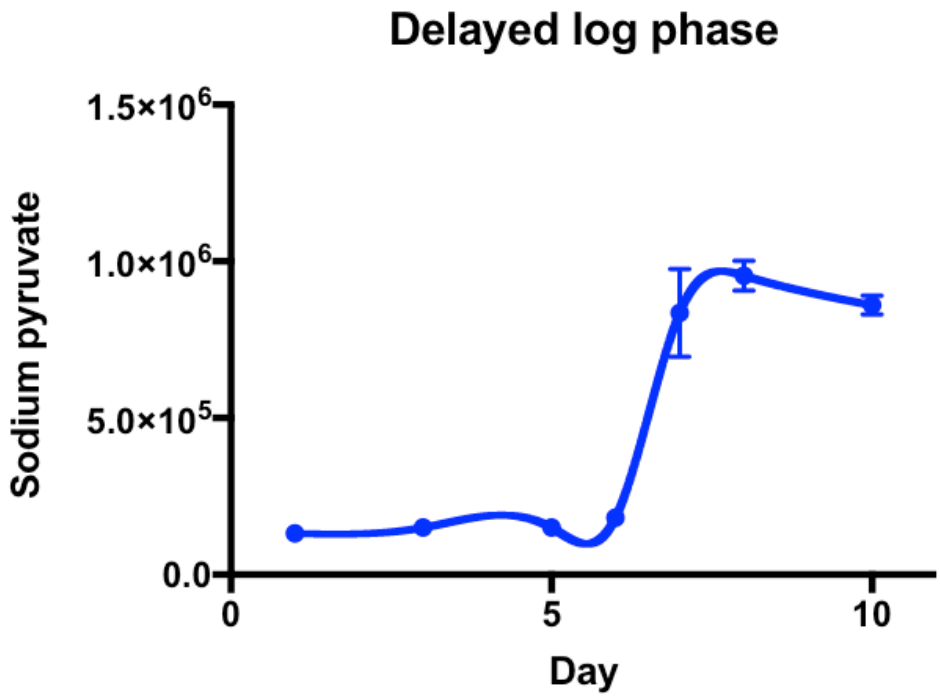


Figure 3.4: Proliferation (± SEM, n=3) of MCF-7 cells exposed to TrypLE™ Express (30 min), highlighting the delay in the log phase to day 6/7.

TrypLE™ Express was demonstrated to separate cells sufficiently following 10 min exposure, thus, because it is gentler on the cells it was continued to be used for cell maintenance (Section 2.2.3.2.). However, when observing the cell suspension under a microscope, the TrypLE™ Express did not sufficiently separate the cells, thus a single cell suspension could not be achieved. Additionally, when cells were seeded using the TrypLE™ Express solution in the protocol, high variability was observed between replicates (Fig. 3.5). Therefore, the trypsin protease solution was used to prepare a single cell suspension for cell seeding (Section 2.2.4.). However, the cells were much more closely monitored under the microscope when exposed to trypsin protease solution to minimise any effects of prolonged exposure such as a delayed log phase or low adherence due to the breakdown of cell surface adhesion molecules. When the cells had completely dissociated from the flask, a single cell suspension could be achieved using the force from expelled liquid in the automatic pipette.

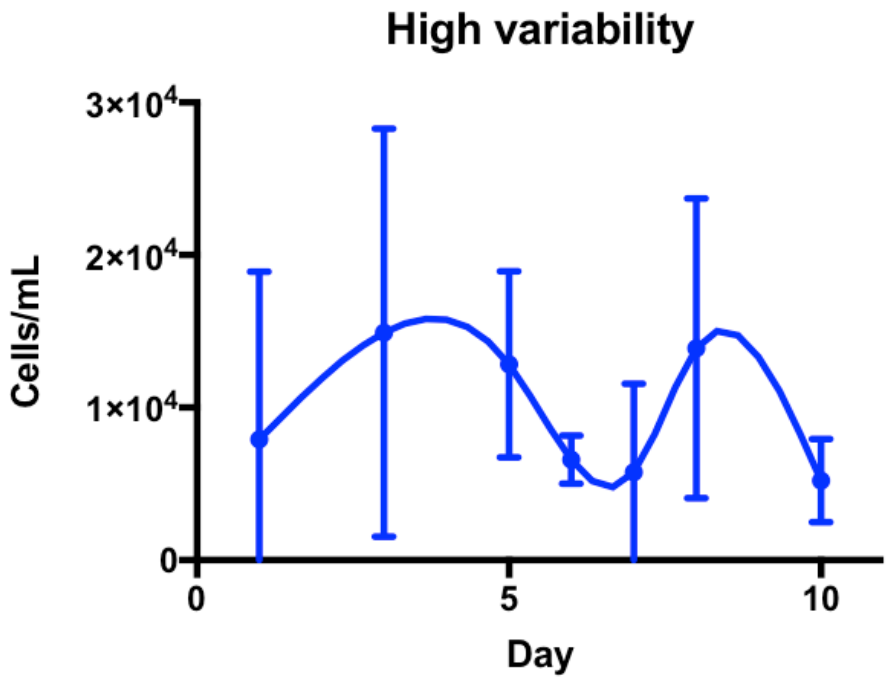


Figure 3.5: Proliferation (± SEM, n=3) of MCF-7 cells exposed to TrypLE™ Express, highlighting the variability cause by prolonged exposure (i.e. 30 min).

3.4. Discussion

It is clear, even from a brief literature search, that there are many different cell culture systems used around the world. This is not surprising when one considers the deviation of the MCF-7 cell line since it was first isolated in 1973. Although, remarkably, the cell line has stabilised over the last 45 years the ability of MCF-7 cells to adapt and evolve over time, this is a concern in the context of historical data from MCF-7 cell experiments. This has led to an difficulty to replicate results in MCF-7 variants across laboratories which has caused concern for people using the MCF-7 cell line for their research (Lorsch*, et al.*, 2014). However, from the perspective of my work the MCF-7 cells still responded to an E2 stimulus as anticipated. In this section of my research I wanted to identify the best culture medium for my MCF-7 model system – this was achieved.

3.4.1. Effect of Culture Medium on MCF-7 Cell Proliferation

Sufficient concentrations of nutrients in the medium are prerequisites for cells to behave consistently. Some cell types require higher concentrations of nutrients than others, depending on their metabolic activity and proliferation rate. Therefore, it is not surprising that MCF-7 cells responded differently to each of the culture media; with cells cultured in MEM culture media having the slowest growth response and low adherence to the culture flask.

MEM culture medium is the least supplemented of the three tested, based on basal medium Eagle (BME). Non-essential amino acids, which MCF-7 cells can biosynthesise, are not included in the MEM formulation. DMEM is modified to include additional non-essential amino acids serine and glycine. MCF-7 cells grown in DMEM culture medium proliferated at a higher rate compared to MEM, which suggests the addition of glycine and serine was necessary for cell growth and proliferation. However, the RPMI-1640 culture media had a positive effect on proliferation compared to MCF-7 cell cultured in both MEM and DMEM, while a high level of adherence to the culture flask was also observed. One of the most significant differences between MEM/DMEM and RPMI-1640 is the addition of eight NEAAs to RPMI-1640. The NEAAs are important in the cell's intermediary biochemistry (see Fig. 3.6) and by extension are important in cell health and proliferation. In general, NEAAs can be biosynthesised in cells (Yao*, et al.*, 2017), thus, they are not included in some basal media such as MEM. It is likely that MCF-7 cells cannot produce sufficient amounts of NEAAs in the cultured state to support proliferation, thus rely on high concentrations of NEAAs in the culture media. The addition of NEAAs in the RPMI-1640 appears to ensure more favourable culture conditions and thus might alleviate the biosynthetic pressure of the cells. Interestingly, the majority of methods that use MEM or DMEM to culture MCF-7 cells add NEAAs (Yao*, et al.*, 2017), highlighting the importance of correct nutrient supplementation for MCF-7 cell culture.

Glutamine, one of the essential amino acids, is an energy source in mammalian cells in culture, in addition to being required for protein synthesis. The glutamine requirements for cell culture are ~3-40 fold greater than those of other amino acids (Eagle, 1959). However, ammonia is released into the culture media as a result of consumption of glutamine by the cells where glutamine undergo the chemical decomposition to pyrrolidonecarboxylic acid. Ammonia is cytotoxic to cells and has been shown to inhibit cell growth (reviewed by (Schneider*, et al.*, 1996)), however, for many years glutamine has been known to be a major energy source for mammalian cells grown in culture (Yao*, et al.*, 2017). It's possible that cell death seen after long term (e.g. 10 days or more) is related in part to depleted glutamine in the culture medium and the production of ammonia metabolism which would be cytotoxic

3.4.2. Effect of Sodium Pyruvate and Glucose Supplementation of MCF-7 Cell Proliferation

In general, the genetic differences between normal and cancer cells lead to significant phenotypic differences; therefore, an altered metabolic phenotype would be required for the normal function of the cancer cell, thus some metabolic precursors are necessary for increased proliferation, providing a growth advantage over normal, non-cancerous cells (DeBerardinis, 2009). From the results, it is clear that glucose did not support the replication and proliferation of the MCF-7 cells, whereby, no change in proliferation was observed between glucose and non-glucose supplemented medium. Although the medium used in this experiment was DMEM which lacks NEAAs; however, one would expect the glucose present in the culture medium to be sufficient to support increased proliferation. Interestingly, when MCF-7 cells were cultured in sodium pyruvate supplemented RPMI-1640, increased proliferation occurred compared to cells exposed to RPMI-1640 without sodium pyruvate supplementation. Pyruvate is a key metabolic intermediate (see Fig. 3.6). It is biosynthesised from glucose through glycolysis and can supply energy to cells through the tricarboxylic acid (TCA) cycle when oxygen is present (Berg*, et al.*, 2002). Without the constant supply of metabolic energy, the frequent replication characteristic of cancer cells would not be possible.

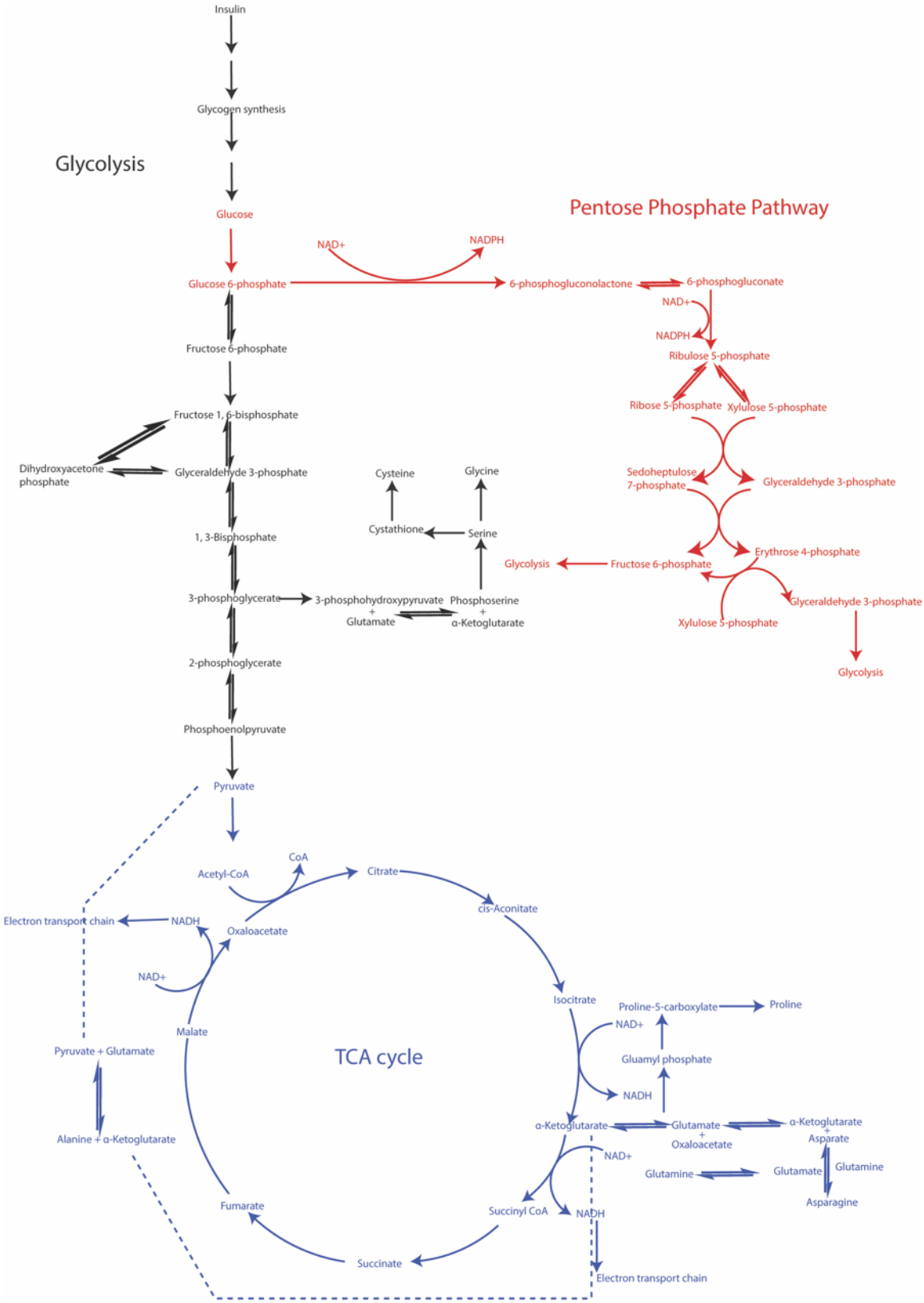


Figure 3.6: Simplified schematic of a cell's metabolic pathways: glycolysis, the citric acid (TCA) and pentose phosphate pathway and the role in of these pathways in amino acid synthesis. The pentose phosphate (red) and TCA (blue) demonstrates how glucose and pyruvate supplementation could circumvent the biosynthesis of glycine, serine and cysteine in MCF-7 cells.

The results from the experiments describe above support those of Diers and colleagues (2012) who also observed rapid proliferation in MCF-7 cells in the presence of pyruvate, compared to cells cultured in glucose-containing medium. The culture media used in these studies was DMEM without NEAA supplementation, which suggests even though my studies were carried out in two different culture media, the presence of the NEAAs did not influence the outcome (Diers*, et al.*, 2012). Clearly, glucose and thus glycolysis, is not sufficient to support rapid proliferation in MCF-7 cells compared to mitochondrial metabolism. However, glycolytic metabolism can provide a growth advantage through several different mechanisms (reviewed in (Kim*, et al.*, 2006)). These include generating ATP at a faster rate than oxidative phosphorylation, providing biosynthesis substrates needed for rapid proliferation (e.g. NADPH and ribose-5-phosphate) and supporting cell growth under hypoxic conditions.

Interestingly, a study conducted by Drabovich and colleagues (2012) showed elevated expression of fructose-1, 6-bisphosphatase (FBP1), fructose-1, 6-bisphosphatase-2 (FBP2) and glucose-6-phosphate dehydrogenase (G6PD) in MCF-7 cells compared to a non-cancerous breast cell line (MCF-10A) (Drabovich*, et al.*, 2012). The elevation of these enzymes increases glucose flux from glycolysis to the pentose phosphate pathway (PPP), redirecting cellular energy metabolism toward increased biosynthesis (Jiang*, et al.*, 2011). This likely explains the low induction of rapid proliferation by glucose-supplemented culture media, with glycolysis being redirected into the PPP. Therefore, even if MCF-7 cells are cultured in a high glucose containing medium, the pathway could be redirected to drive synthesis of ribose 5-phosphate, a precursor for the synthesis of nucleotides and other macromolecules, leaving glycolysis with minimal substrates to produce pyruvate. It is not surprising that the cells drive the production of ribose 5-phosphate (see Fig. 3.6) considering their rapid proliferation and high dependence on the synthesis of nucleotides, and thus DNA synthesis. This pathway favourability could also explain the induction of rapid proliferation of cells cultured in pyruvate containing media, where the exogenous pyruvate to the culture medium bypasses glycolysis, and thus the redirection into PPP, allowing for effective biosynthesis of the TCA macromolecules.

Interestingly, an emerging concept in the field of cancer metabolism suggests the importance of mitochondrial metabolism, particularly tricarboxylic acid (TCA) activity, in providing intermediates required for the biosynthesis of cellular macromolecules (e.g. fatty acids, NEAAs). It has been shown that metabolism of mitochondrial substrates such as glutamine and pyruvate are necessary to support the rapid proliferation of multiple cells types, and a

functional link between mitochondrial respiration and proliferation capacity (Pike*, et al.*, 2011, Weinberg*, et al.*, 2010). This suggests that pyruvate might be required for MCF-7 cells to have the necessary energy reserves thought to be critical for effective cell function in adverse conditions (Dranka*, et al.*, 2010, Jekabsons*, et al.*, 2004).

It is likely that the energy required to produce the NEAAs is directed towards the biochemistry associated with replication if the NEAAs are provided in the culture medium – indeed NEAAs could be regarded as essential for cancer cells which divide frequently. This also highlights the need for NEAAs media supplementation (see Section 3.4.1.). In addition, glycine and serine are biosynthesised from glucose via glycolysis (Fig. 3.5) which further supports the need for NEAA supplementation. If glucose was supplemented to give the cells the metabolic wherewithal to biosynthesise NEAAs the metabolic flux would redirect glucose to the PPP which would not achieve the goal NEAA biosynthesis. On the other hand, if pyruvate is supplemented it directly enters the TCA and bypasses glycine and serine biosynthesis. In short glucose does not provide the cell its NEAAs needs and pyruvate bypasses the biosynthesis of two key NEAAs and therefore NEAAs is required by MCF-7 cells in culture.

3.4.3. TrypLE™ Express and Trypsin Protease Dispersal of MCF-7 Cells

Trypsin or TrypLE™ Express cleaves peptide bonds with lysine and arginine on the c-terminal side unless they are followed by proline. They are the most common enzyme preparations in tissue culture used to release adherent cells from culture flasks (i.e. the matrix) and/or each other (Heng*, et al.*, 2009). Cell-cell and cell-matrix interactions depend on adhesion molecules such as cadherin which, in turn, relies on calcium for activity. Cadherin is important in the formation of adhesion junctions which mediate cell-cell and cell-matrix interactions. Since Ca^{2+} and Mg^{2+} are important for growth and development and biochemistry in general, this of course facilitates adhesion processes. In the context of cell culture, unfortunately trypsin is inhibited by Ca^{2+} Mg^{2+}, for this reason EDTA is usually added to trypsin solutions because it chelates Ca^{2+} and Mg^{2+} which facilitates trypsin activity (Dainiak*, et al.*, 2007).

This study compared the dissociation of adherent MCF-7 cells with either TrypLE™ Express or trypsin protease, the most commonly used enzyme for dissociation of cultured cells *in vitro*. The TrypLE™ Express is designed to be gentler on cells, and this was evident from the

experiments. The TrypLE™ Express exposed cells required a longer exposure to get the desired dissociation. However, it did not separate the cells to get a single cell suspension required for the experiments as described (Section 2.2.4.). However, prolonged exposure (30 min) to the trypsin protease solution affected cell proliferation by decreasing initial adhesion of the cells to the flask and delaying the log phase for up to three days. This is not surprising considering the enzymes mechanism of action cleaving peptide bonds. Prolonged exposure leaves the cell surface stripped of proteins which would at the very least change the morphology of the cell or could even cause cell death.

When TrypLE™ Express is used in routine cell culture, it effectively removes the cells from the matrix but to some extent reduced cell-cell adhesion. The degree cell-cell and cell-matrix dissociation was sufficient for the purposes of routine passage. On the other hand, trypsin was very effective in reducing cell-cell and cell-matrix adhesion usually resulting in liberation of single cells; this is required for MCF-7 cell experiments. Therefore, TrypLE™ Express was used for routine passaging and trypsin was used for experiments where a single cell suspension was required.

3.5 Concluding Remarks

The results presented in this Chapter highlights the importance of understanding the biochemistry of cultured cells. In the MCF-7 cell line it is clear that the metabolic requirements are vast, and therefore, important in developing and understanding the culture system. Whilst MCF-7 cells have been fundamental in breast cancer research, limited information is available on the rationale behind the multitude of culture systems used. Therefore, this chapter provides insights into culturing the MCF-7 cell line which is important when using them for breast cancer research.

Chapter 4 MCF-7 Exposure Studies

4.1 Introduction

The ER evolved in a pristine environment in which it developed a highly specific relationship with estrogens, particularly E2. Sequence analysis indicates that the ER evolved before other nuclear receptors such as AR and PR, with an ancestral ER identified in basal animals (e.g. sponges, coral, etc.) but not plants, yeast and bacteria (Baker, 2005, Baker, 2008, Bertrand, *et al.*, 2011, Bertrand, *et al.*, 2004, Bridgham, *et al.*, 2010, Sladek, 2011). However, with the industrial revolution came the development of a myriad of chemicals, many of which would be found to be E2 mimics. In turn, the ER was found to be surprisingly promiscuous with the accommodation of a broad array of ligands at the LBC binding site. The development of chemicals did not stop there with millions of chemicals being registered every year many of which have unknown estrogenicities. For example, in 1995 there were 211,934 chemical abstract service (CAS)-registered chemicals, this increased to 88, 758, 285 by 2006 (Binetti, *et al.*, 2008). Improvements in industrial productivity over the last 70 years have meant that large quantities of structurally diverse, persistent chemicals have been released into the environment (Bitman, *et al.*, 1968, Colborn, *et al.*, 1993). Over 800 compounds are known to disrupt the endocrine system, with over 160 of these compounds identified as being xenoestrogens (Brody, *et al.*, 2006, Brody, *et al.*, 2003, Brody, *et al.*, 2007). However, this number is likely a vast underestimate with only a select number of chemicals being tested to date, many of whom have been selected based on toxicological evidence.

Many xenoestrogens are found in the environment and foods; however, their *in vivo* predictability has come under scrutiny with the development of different techniques and tools used for assessing their estrogenic effects (Graham, 2012, Thomson, 2005). The major routes of exposure for humans are assumed to be via the regular diet of food containing natural phytoestrogens, compounds leaching from food wrapping materials, and residues of pesticides, as well as personal care products such as wash off products (shampoos and soaps) that are used regularly, often daily (Aurela, *et al.*, 1999, Erlund, *et al.*, 2002, Franke, *et al.*,

1998, Saito*, et al.*, 2002). Thus, a normal diet and daily habits already result in exposure to complex mixtures of xenoestrogens, resulting in systematic circulation in the body (van Meeuwen, 2008). Therefore, while xenoestrogens are generally found at low concentrations in the environment, the overall impact of xenoestrogen mixtures could lead to biological effects that are at least additive. There is wide concern about the increasing exposure of humans to xenestrogens. In this respect, several xenoestrogens have been suggested to pose a possible risk to humans and concern has been raised because of a suggested link with breast cancer (Siddiqui*, et al.*, 2016).

Many common environmental chemicals are mammary gland carcinogens in animal studies, activate relevant hormonal pathways, or enhance mammary gland susceptibility to carcinogenesis. The long latency and multifactorial aetiology of breast cancer have made evaluation of these chemicals as possible carcinogens in humans challenging (Rodgers*, et al.*, 2018). However, the possibility that the bioaccumulation of xenoestrogens may cause breast cancer has been raised by past epidemiological studies on environmental and occupational exposure. Associations have been reported between breast cancer risk and serum or fatty tissue levels of 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) or 2,2-bis-(*p*-chlorophenyl)-1, 1, 1-trichloroethane (DDT), sometimes linked to ER positive tumours (Djordjevic*, et al.*, 1994, Falck*, et al.*, 1992, Wolff*, et al.*, 1993). Major risk factors for breast cancer include sex, age, genetic predisposition, reproductive history and lifetime exposure to breast tissue estrogens as well as modifiable risk factors such as obesity, lack of physical activity and high alcohol intake (see Section 1.5.3.). Exposure to xenoestrogens early in life is also thought to alter breast development and increase adult susceptibility to breast cancer. For example, the synthetic estrogen diethylstilbestrol (DES) has been associated with breast cancer after age 40 in a U.S. cohort of women who were exposed *in utero* (Hoover*, et al.*, 2011). However, the age-standardised breast cancer incidence rates are increasing in some developed and developing countries, which cannot be explained by these major risk factors (DeSantis*, et al.*, 2015, WHO, 2013).

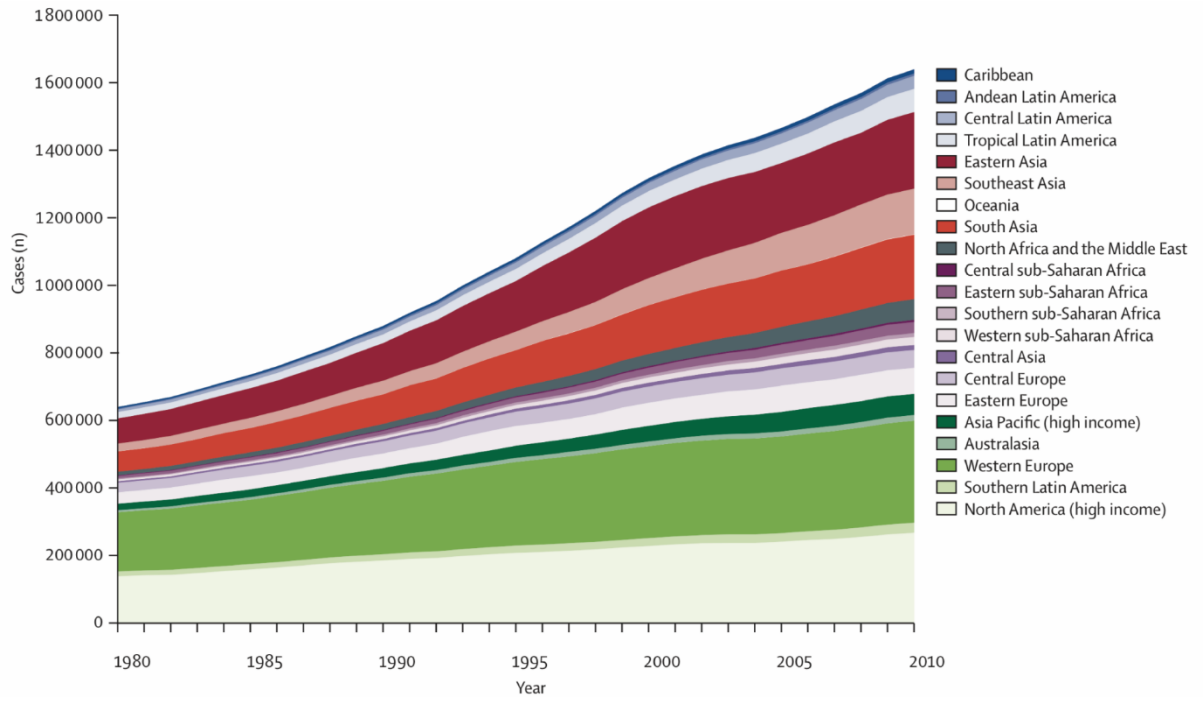
The questions posed by Safe and colleagues (2018) summarised the current controversy in the field: “The important toxicological question concerning these chemical xenoestrogens is whether they differ only in potency which is governed by their intrinsic estrogenicity and bioavailability based on their metabolism and pharmacokinetics. On the other hand, since the estrogenic activity of these compounds is ER dependent, is the estrogenic activity of structurally diverse xenoestrogens more unique and not necessarily governed by intrinsic ER

binding affinities and pharmacodynamics factors?" Indeed, results from X-ray crystallographic analysis clearly demonstrate that both ER agonists and ER antagonists differentially bind the ER and induce compound-specific changes in the bound complex, leading to different transactivation induction (Bernstein, 2002). Not only that, they also have different ER isoform preferences such that genistein and other phytoestrogens preferentially bind ERβ, while synthetic xenoestrogens, such as EE2, preferentially bind ERα (see Section 1.4.). This indicates that the potential associated risk of xenoestrogen combination and breast cancer, are very much dependent on the mixture composition. However, not all xenoestrogens have negative implications for human health, in fact, phytoestrogens (plant derived xenoestrogens) are well known to exhibit a litany of health benefits including lower risk of osteoporosis, heart disease and breast cancer (Patisaul*, et al.*, 2010).

4.1.1. Phytoestrogens

Humans are exposed to high concentrations (e.g. µM concentrations) of isoflavones in their day-to-day lives. Isoflavones are a good indicator of phytoestrogen intake because isoflavones are the main component of consumed phytoestrogens (e.g. genistein, daidzein and equol). A meta-analysis by Lim and Shaw (2016) shows that isoflavone intake in China varies from 5.7 mg/day to 89.3 mg/day, while in the USA intakes vary from 0.8 mg/day to 13.7 mg/day. Intake data from the USA likely represent Western diets, while Chinese data likely represent Asian diets. Interestingly, data from both diets are converging, with an increase in Western diets to 13.7 mg/day and a significant decrease in Asian diets to 15.6 mg/day (Lim*, et al.*, 2016). Interestingly, Asian populations historically had lower rates of breast cancer compared to Western populations, however, breast cancer incidence is increasing in Asian countries which has been linked to soy consumption (Adlercreutz*, et al.*, 1997). Soy is the corner stone of a traditional Asian diet, an observation which had long fuelled the widely held belief that consumption of soy reduces the risk of breast cancer. This makes is sound really simple, however, it is far more complex because now we know more about phytoestrogen intake it looks like the breast cancer incidence is not only related to soy consumption. Phytoestrogens, especially genistein and daidzein, are largely attributed as the compounds responsible for the reduced breast cancer risk and have particularly gained a lot of interest in the area since the 1980's after findings of epidemiological studies that compared Western and Asian diets. Results indicate that a diet with high phytoestrogen content may give some prevention against predominant Western diseases such as breast cancer (Adlercreutz, 2002, Cassidy*, et al.*, 2000). However, with a significant decrease in soy consumption and rising breast cancer incidences in Asia, one could speculate that there may

be a negative relationship between soy consumption and breast cancer incidence. Age-standardised incidence is going up in some countries around the world (Fig. 4.1) which could be related to dietary xenoestrogen exposures, however causation cannot be inferred from descriptive data alone. It is much more complicated that simple soy consumption; this graph shows developed and developing nations where it so happens that some developing countries eat a lot more soy than the developed countries. However, there are other factors such as differences in regulatory status of chemicals and therefore, exposure of populations to environmental chemicals, such as pesticides or components of cosmetics can vary greatly



between countries. Thus, soy is not the single the determinant in breast cancer risk around the world.

At present, several mechanisms underlying this possible beneficial effect of phytoestrogens have been suggested: (i) specifically binding and activating ERβ more than ERα, although generally with less affinity than E2 (Kostelac*, et al.*, 2003, Kuiper*, et al.*, 1997, Kuiper*, et al.*, 1998), (ii) acting as a SERM (selective estrogen receptor modulator) (Diel*, et al.*, 2001), (iii) reducing ER expression (Chen*, et al.*, 2003, Miodini*, et al.*, 1999, Sathyamoorthy*, et al.*, 1997), (iv) competing as agonist with other endogenous estrogens on the ER, (v) inducing apoptosis (Pagliacci*, et al.*, 1994), (vi) lowering estrogen levels in circulation (Ibarreta*, et al.*,

Figure 4.1: Incidence of breast cancer in individuals grouped by geographical area between 1980 and 2010 (Forouzanfar*, et al.*, 2011).

2001), (vii) reducing angiogenesis and tumour invasiveness (Chen, *et al.*, 2003, Magee, *et al.*, 2004), and (viii) scavenging radicals (Brownson, *et al.*, 2002, Nijveldt, *et al.*, 2001, Zand, *et al.*, 2000). Clearly, there are many possible mechanisms of action attributed to the possible beneficial effects of phytoestrogens; thus, understanding their role in xenoestrogen endocrine disruption is important in unravelling the cocktail effect of xenoestrogens as breast cancer risk factors.

4.1.2. Xenoestrogens as Endocrine Disruptors

There are several mechanisms of disruption that can affect estrogen signalling (Fig. 4.2). The mechanisms can be categorised as ER mediated (e.g. genomic and non-genomic signalling) and non-ER mediated (e.g. competition at SHBG). Xenoestrogens are able to interfere with multiple steps illustrated in Figure 4.2 that evidently will lead to altered gene expression. To further complicate matters xenoestrogens may simultaneously act via more than one mechanism (see labelled numbers 1-12 in Figure 4.2). For example, a xenoestrogen may interfere with E2 biosynthesis, compete for binding to the SHBG and compete for binding at the ER LBC compounding the estrogenic effects. For these reasons, a phenotypic definition of xenoestrogens as endocrine disruptors is difficult to provide. Thus, with such a complex nature, regulation of the use and exposure of xenoestrogens has been limited to individual compounds, with mixtures proven to be too complex to safely regulate.

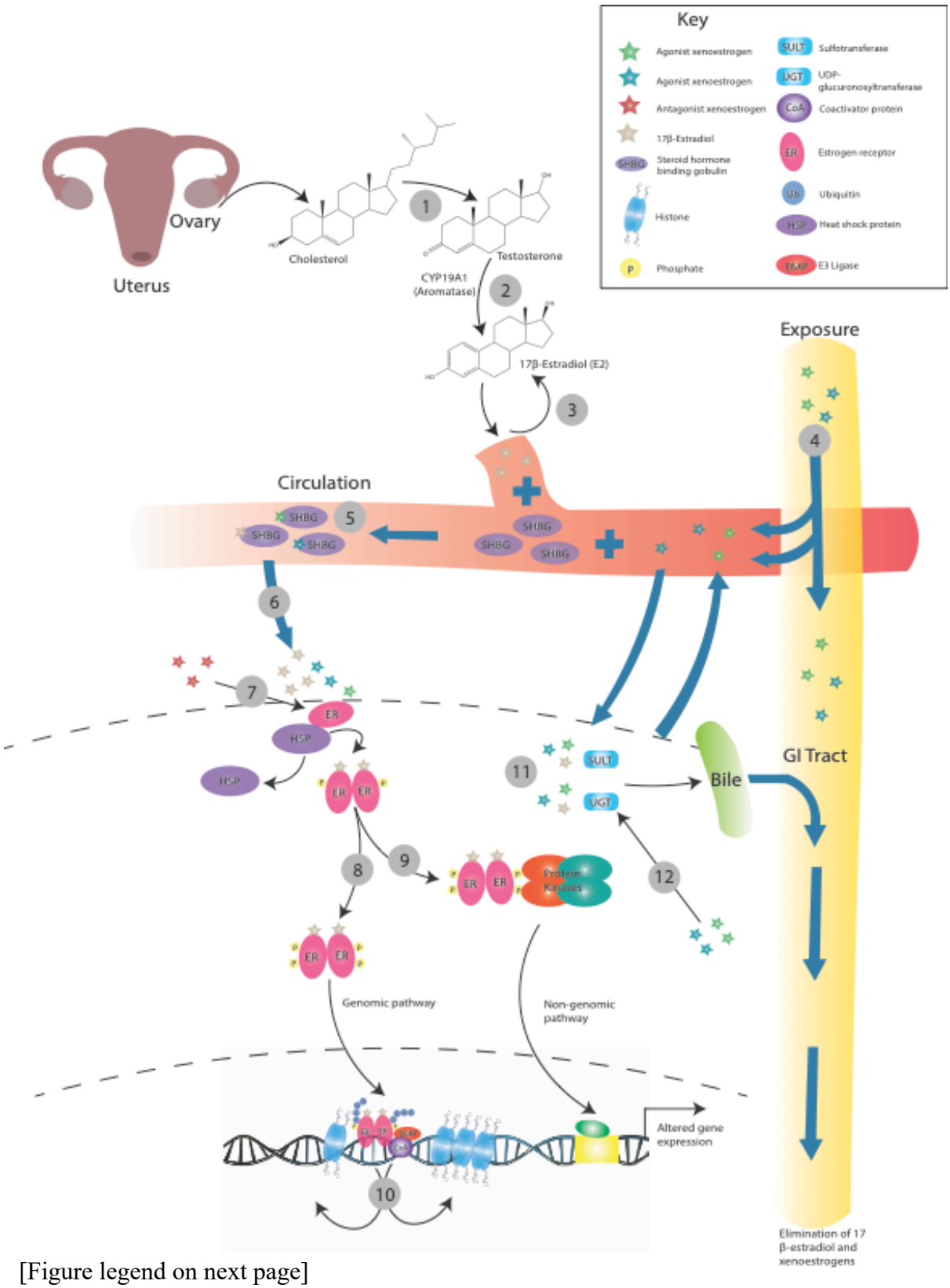


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Figure 4.2: Potential mechanisms of xenoestrogen endocrine disruption. (1) Up/down regulation of testosterone biosynthesis leads to changes in E2 biosynthesis. (2) Up/down regulation of aromatase leads to altered E2 biosynthesis in the ovaries. (3) Changes in E2 feedback mechanisms leads to altered E2 biosynthesis. (4) Xenoestrogens gastrointestinal (GI) tract metabolism leads to a more or less potent xenoestrogen. (5) Competition for hormone carrier proteins (e.g. steroid hormone binding globulin, SHBG) increases the concentration of free E2. (6) Agonist xenoestrogens compete for ER binding altering genomic (8) and non-genomic (9) signalling pathways. (7) Antagonist xenoestrogens compete for ER binding altering genomic (8) and non-genomic (9) signalling pathways. (10) Changes in epigenetic modification (e.g. transcriptional and post-translational) leads to altered gene expression. (11) Altered E2 metabolism by competition for phase II enzymes (e.g.sulfotransferase (SULT) and uridine 5'-diphospho- glucuronosyltransferase (UGT)) alters free E2 concentrations. (12) Up/down regulation of phase II metabolic enzymes alters free E2 concentrations (concept of the GI tract, circulation and bile sections of this diagram were taken from (Kodavanti*, et al.*, 2010).

Thus, in order to fully understand the estrogenic or anti-estrogenic activities of xenoestrogens, studies will have to consider multiple mechanisms of actions of ER-xenoestrogen complexes. Risk assessment of these compounds is complex and dependent on all the variables mentioned above. Moreover, since individual xenoestrogens exhibit unique biologies, the overall impact of mixtures of these compounds may not be additive. These factors highlight the challenges faced by scientists and regulators in addressing the health risks and benefits of estrogenic compounds (Safe*, et al.*, 2018).

In addition, assessing xenoestrogen cocktails, particularly in women who are most at risk of developing breast cancer, is not as straightforward as it first appears. Circulating E2 levels vary significantly throughout a woman's life which can impact on the effects of xenoestrogen mixtures; therefore, the proportion E2 contributes to a mixture varies depending on the day in the estrus cycle and stage of development (e.g. pre-puberty, post-menopausal, etc.) of a woman, thus it is important to consider the circulating E2 levels when assessing xenoestrogen cocktail risk. Women who are of child-bearing age have high circulating E2 levels (20-250 pg/mL) compared to postmenopausal women or pre-pubertal girls who have low circulating E2 levels (<20 pg/mL). Such varying concentrations of E2 might significantly impact on the activity and estrogenic effect xenoestrogen exposures might have at the individual level.

4.1.3. Current Understanding of Xenoestrogen Cocktails

MCF-7 cells, which are ER+ve, are often used to study xenoestrogen, as they exploit the principle that MCF-7 human breast cancer cells proliferate in the presence of chemicals that directly or indirectly activate the ERs. In previous MCF-7 cell proliferation studies, combinations of benzylbutyl phthalate, BPA, DDE and hexachlorobenzene and E2 with either BPA or pentachlorophenol have been reported to give a synergistic proliferative

response (Suzuki*, et al.*, 2001). In contrast, mixture experiments with nonyl and octylphenol and E2 and benzylbutyl phthalate have been reported to negatively affect MCF-7 cell proliferation (Rajapakse*, et al.*, 2004). Additionally, multiple studies by Kortenkamp and colleagues have shown xenoestrogen mixtures to be additive in MCF-7 cell studies (Evans*, et al.*, 2012, Kortenkamp, 2007, Kortenkamp, 2008, Kortenkamp*, et al.*, 2007, Payne*, et al.*, 2000, Rajapakse*, et al.*, 2002, Rajapakse*, et al.*, 2004, Silva*, et al.*, 2002). Additivity in the context of this work describes the case in which chemicals act together to produce effects without enhancing or diminishing each other's actions.

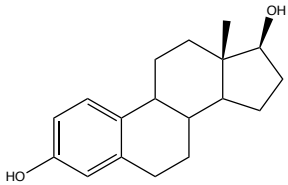
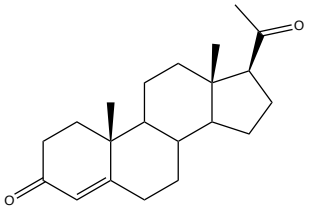
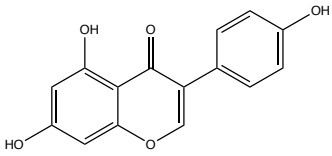
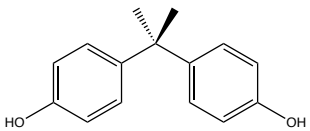
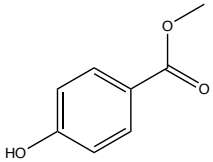
The identification of xenoestrogens in the environment, coupled with human exposures to these compounds, has generated public, regulatory and scientific concern regarding their potential hormonal toxicity resulting in human health risks and risks to wildlife. While there has been an increased interest in xenoestrogens, they still remain regulated individually due to the complexity of their mechanisms of endocrine disruption. In addition, the hypothesis that environmental/dietary xenoestrogens may increase breast cancer incidence is controversial and the significance of these compounds on human health is not resolved. However, recent research has started to propose the involvement of xenoestrogens via interfering with estrogenic pathways, as one of the causes of increasing incidence rates of breast cancer (WHO, 2013).

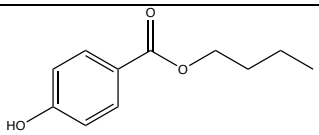
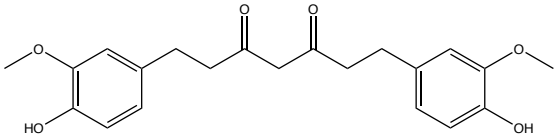
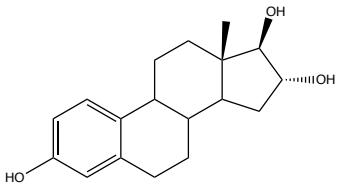
Clearly, exposure to xenoestrogens is far more complex that the concept of simple additivity; thus, the overall effects of mixtures still remains inconclusive. However, in view of the suggested adverse effects of xenoestrogens on human health and wildlife, it is important for the risk assessment process to establish the types of mixture interactions that may occur with exposure to combinations of these natural and synthetic xenoestrogens. The aim of this chapter is to assess xenoestrogen combinations to determine the effect on MCF-7 cell proliferation, in order to understand the mixture effects in a breast cancer model system. Phytoestrogens are present in food at fairly high concentrations, thus we are all exposed to them every day. There is also a very large literature on their biochemistry and toxicological effect; for example, the mixed proliferative/anti-proliferative effects of genistein in cultured breast cancer cells. This makes them interesting candidates for study. Therefore, it was reasonable to included them as a major part of the study.

4.2. Experimental Approach

Xenoestrogens (Table 4.1) were selected for MCF-7 exposure studies based on previous published studies which showed a high likelihood of women being exposed throughout their day-to-day lives. There was a focus on phytoestrogens because (1) they are present in a significant proportion of foods people consume every day, (2) they are likely to induce simple additivity, and (3) they have potential to exert breast cancer preventive effects. Two studies were carried out in order to assess xenoestrogen mixtures: a dose response study which coupled eight increasing concentrations of xenoestrogens with either E2 or another xenoestrogen, and 10-day growth curve studies which examined the impact of exposure to a combination of xenoestrogens where the individual components of the mixture were selected at fixed concentrations.

Table 4.1: Xenoestrogens selected to be studied in MCF-7 exposure studies: E2, EE2, genistein, BPA, methylparaben, butylparaben, tetrahydrocurcumin, kaempferol and estriol.

Estrogen/xenoestrogen	Structure
E2	
EE2	
Genistein	
BPA	
Methylparaben	

Butylparaben	
Tetrahydrocurcumin	
Estriol	

4.2.1. MCF-7 Dose Response Studies

Initially, MCF-7 dose response experiments were carried out to determine the combination concentration for the 10-day growth experiments (see Section 2.2.6.). Cells were exposed to increasing concentrations (n=8) of each of the eight xenoestrogens with and without E2 (1.0×10^{-11} M) in a 24 well plate. Additionally, methylparaben/butylparaben and E2/BPA/genistein combinations were also studied (Table 4.2). Cells were exposed to the combinations for five days and then counted in triplicate and repeated in triplicate using a Vetriplast cell counting chamber as described (Section 2.2.4.1). The five-day exposure period was chosen based on the time taken for cells to reach the middle of the log phase without any xenoestrogens present (see Fig. 3.3). From the results, a concentration combination for each of the eight xenoestrogen/E2 combinations was selected to be used for 10-day growth curve experiments (Section 2.2.6.). The concentration combination was selected based on the lowest concentration where an effect was seen (e.g. where there was a clear positive or negative effect on proliferation).

Table 4.2: Selected concentrations of each xenoestrogen for MCF-7 dose response experiments. Varied compounds are tested at eight increasing concentrations with a fixed compound added for combination studies. The tick indicates the xenoestrogen combination used in each experiment.

Fixed compounds (M) ▶ Varied compounds (M) ▼	E2 (1.0×10^{-11})	Butylparaben (1.0×10^{-8})	Genistein (1.0×10^{-8})	BPA (1.0×10^{-8})
E2 ($1.0 \times 10^{-8.5}$- 1.0×10^{-12})			✓	✓
E2 ($1.0 \times 10^{-8.5}$- 1.0×10^{-12})			✓	
EE2 ($1.0 \times 10^{-9.5}$- 1.0×10^{-13})	✓			
Genistein ($1.0 \times 10^{-5.5}$- 1.0×10^{-9})	✓			
BPA ($1.0 \times 10^{-5.5}$- 1.0×10^{-9})	✓			
Methylparaben ($1.0 \times 10^{-4.5}$- 1.0×10^{-8})		✓		
Butylparaben ($1.0 \times 10^{-5.5}$- 1.0×10^{-9})	✓			
Tetrahydrocurcumin ($1.0 \times 10^{-6.5}$- 1.0×10^{-10})	✓			
Kaempferol ($1.0 \times 10^{-5.5}$- 1.0×10^{-9})	✓			
Estriol ($1.0 \times 10^{-7.5}$- 1.0×10^{-11})	✓			

4.2.2. MCF-7 10-day Growth Curve Studies

The effects of the xenoestrogen combinations selected from the dose response experiments were examined over a 10-day exposure period. MCF-7 cells were exposed to the selected combination (Table 4.3), and cell proliferation was determined in triplicate (e.g. each well was counted three times and each daily time point was repeated three times) on days zero, 1, 3, 5-8 and 10 using a vetriplast counting chamber (2.2.4.1.). In addition, cells were exposed to a combination of all eight xenoestrogens/E2 and counted in triplicate. The 10-day exposure period was selected because MCF-7 cells reach the plateau/stationary phase at day 10 (see model system chapter Fig. 3.2). Cell numbers were compared at day 6 as this is where the

cell growth stabilised, which was selected based on the time taken to reach the top of the log phase (see Fig. 3.3).

4.3. Results

4.3.1. Individual Xenoestrogen Dose Responses

Each xenoestrogens studied showed a definite dose-response effect (Fig. 4.3). If the concentrations were sufficiently low, then a classic dose-response curve was seen. In most cases it is obvious.

4.3.2. Xenoestrogen Combination Dose Responses

The growth of the MCF-7 cells following exposure to varying concentrations of xenoestrogen combinations is shown in Figure 4.3. Dose responses for the xenoestrogen combinations show differing patterns of response compared to the corresponding individual response experiments. Two clear categories of responses emerged, those that positively increased the MCF-7 proliferative response and those that decreased proliferation. Xenoestrogen combinations that positively affected proliferation included EE2/E2 and BPA/E2, while the remaining combinations negatively affected the proliferative response to varying degrees. Most surprisingly, when comparing the combination responses to the response elicited by exposure of E2 (1×10^{-11} M), the combinations that had a negative effect on proliferation showed a significant decrease in cell number, whereas the combinations that had a positive effect on proliferation only increased the response initially before following a similar trend to the individual xenoestrogen response. Tetrahydrocurcumin and estriol, both in the presence of E2, decreased MCF-7 cell proliferation so much that the response was similar to the control response.

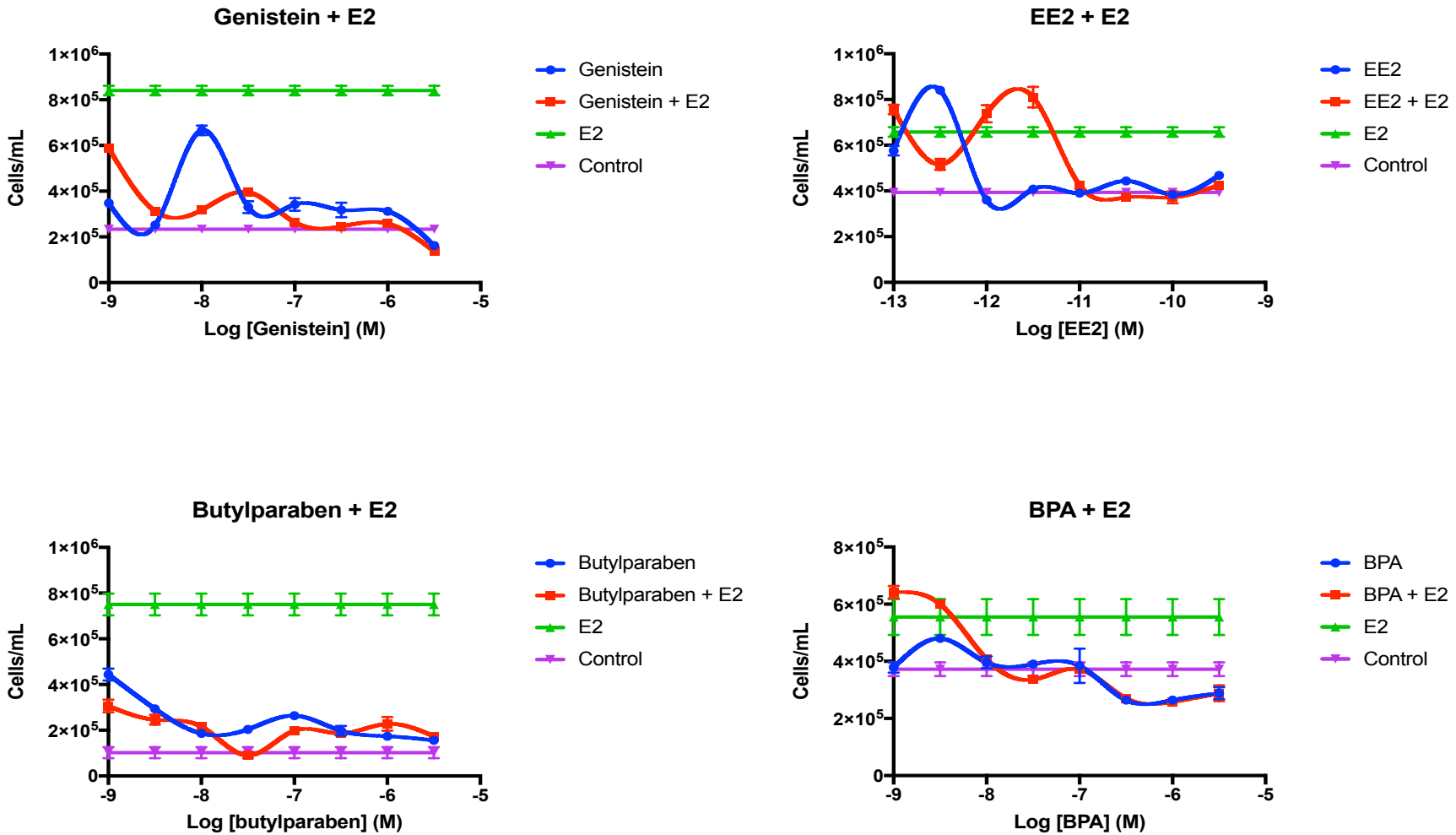


Figure 4.3: also see pages 119 and 120

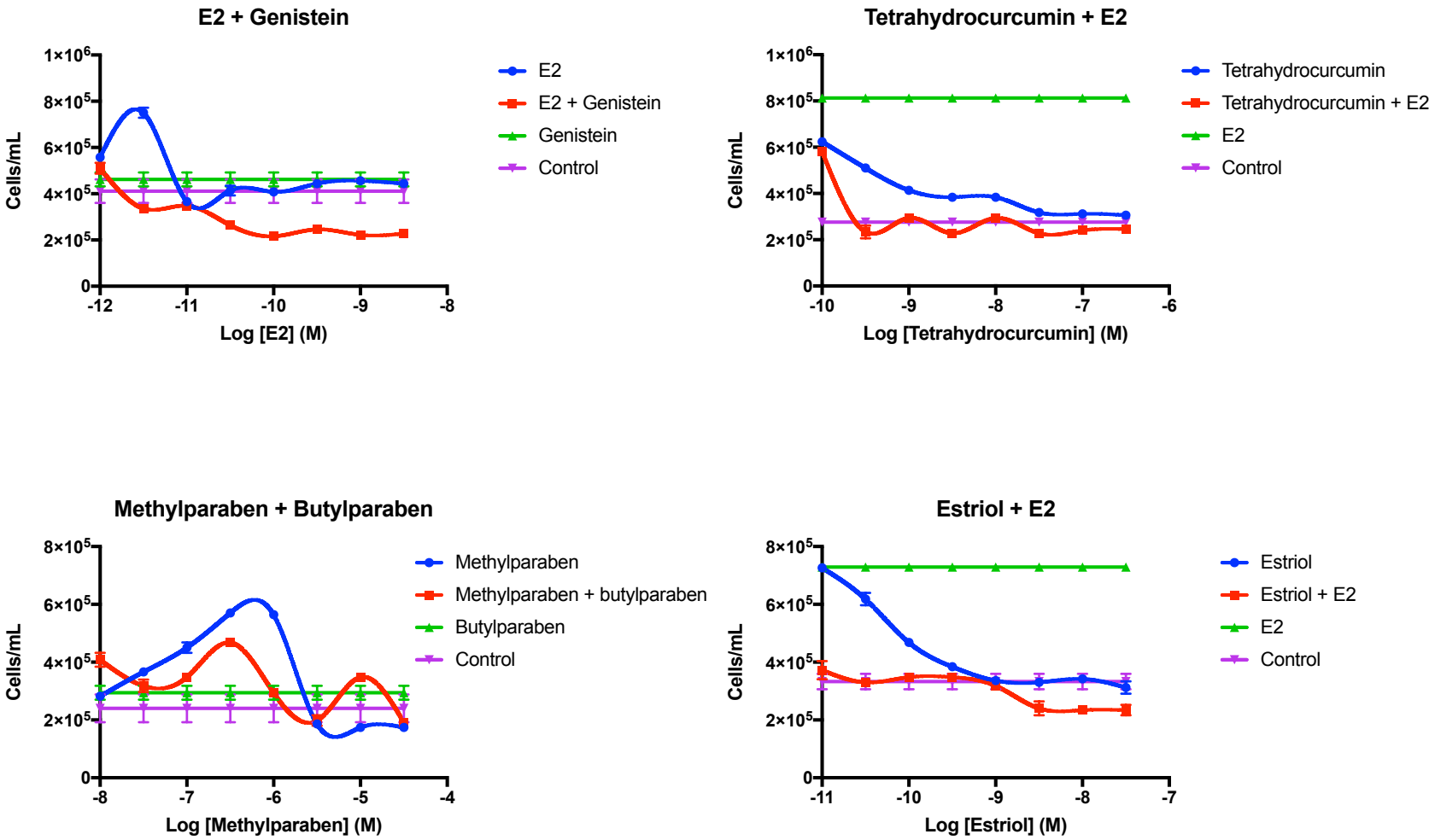


Figure 4.3: also see page 120

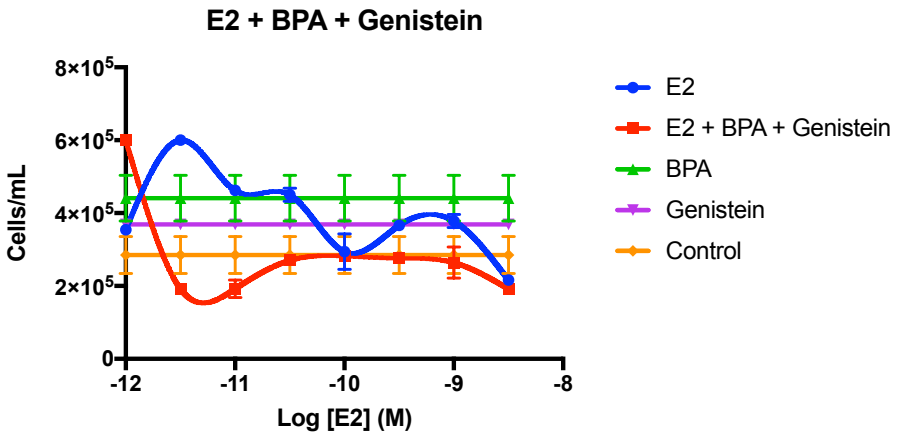


Figure 4.3 (also see pages 118 and 119): MCF-7 proliferation (± SEM, n=3) response to individual and combinations of xenoestrogens. EE2 and BPA combinations exhibited had a positive effect on proliferation at low concentrations, while genistein, butylparaben, E2, tetrahydrocurcumin, estriol and methylparaben combinations negatively affected proliferation compared to individual responses.

From the graphs shown in Figure 4.3, the exposure concentrations of the xenoestrogen combinations were selected for the 10-day growth curve experiments. The concentration at which there was either a definite increase or decrease in the proliferation response was selected for all xenoestrogens except genistein. In the genistein dose response curve there was an unexpected and unexplained peak. However, upon comparison with the docking experiments (Chapter 6) it was clear that the calculated binding energy for genistein did not support the definite increase/decrease observed, whilst they did for all the other xenoestrogens. Thus, because the calculated binding energy for genistein was very similar to butylparaben and BPA, the same concentration was selected for genistein to take account of the unexpected peak. These exposure concentrations are summarised in Table 4.3.

Table 4.3: Exposure concentrations of xenoestrogens to be tested in 10-day MCF-7 growth curve experiments.

Compound	Exposure concentration (M)
Genistein	1.0×10^{-8}
BPA	1.0×10^{-8}
EE2	1.0×10^{-12}
Butylparaben	1.0×10^{-8}
Kaempferol	1.0×10^{-8}
Tetrahydrocurcumin	1.0×10^{-9}
Estriol	1.0×10^{-10}
E2	1.0×10^{-11}

4.3.3. 10-Day Xenoestrogen Combination Growth Curve Responses

MCF-7 cells were grown in culture over 10 days and were shown to have a typical sigmoidal growth curve (Fig. 3.2). Seven individual MCF-7 exposure experiments containing two xenoestrogens followed by 1 combination exposure experiment with eight xenoestrogens were studied (see Figs. 4.4, 4.5 and 4.6). Results were similar to the dose response experiments where two clear groupings emerged, those that had a positive effect on proliferation and those that had a negative effect on proliferation compared to the control.

The EE2 and BPA combination studies again showed additivity in MCF-7 cell proliferation of 21.4% and 16.9%, respectively when compared to the control response at day 6, and an increase of 1.8%-2.4% compared to the respective individual xenoestrogen in the combination. When comparing proliferation induced by these two combinations, both combinations approached the response of E2 with only a 7.1%-15.1% difference. Whether this is biologically significant is uncertain, however, in the context of a cocktail of chemicals when one might be exposed to 20-30 xenoestrogens in a day it might be expected to have a biologically significant effect.

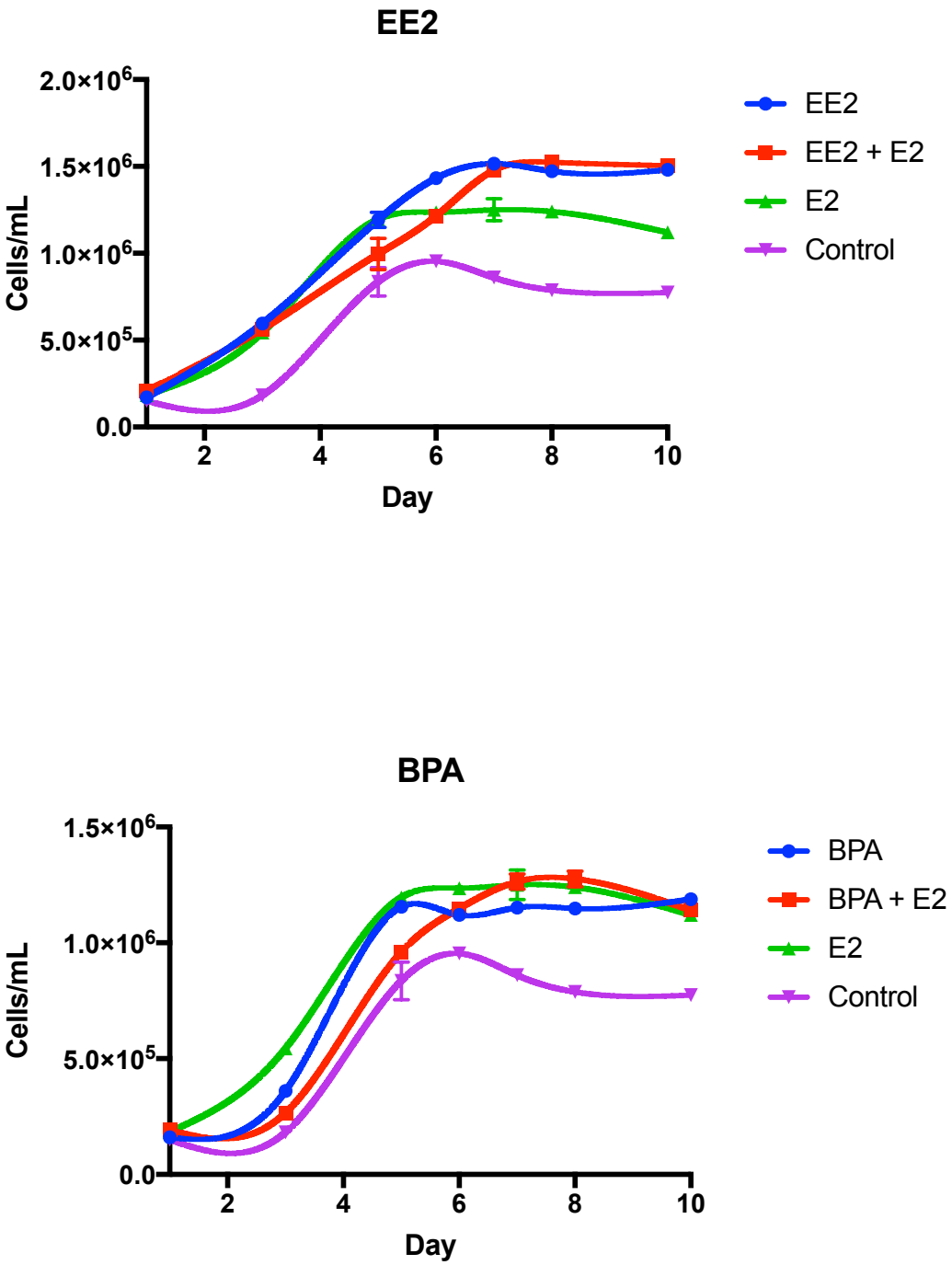


Figure 4.4: MCF-7 proliferation (± SEM, n=3) response to individual and combinations of EE2 and BPA. EE2 positively affected proliferation compared to E2 and the control. BPA positively affected proliferation compared to the control and elicited a similar response to that of E2.

Cell proliferation resulting from treatment with the remaining xenoestrogens (genistein, butylparaben, kaempferol, tetrahydrocurcumin and estriol) in combination with E2 all showed a significant decrease in proliferation. Genistein decreased cell proliferation by 47.4% when compared to E2, while butylparaben decreased cell proliferation by 28.8%. Tetrahydrocurcumin and kaempferol decreased proliferation by 46.6% and 54.8%, respectively, while the only other endogenous estrogen included in this study (estriol) decreased proliferation by 48.9%. Interestingly, when the combinations are compared with the control growth curve at day 6, all combinations negatively affected proliferation with a decrease by 31.0%-38.5%, except for the butylparaben combination which caused a 7.8% decrease (Table 4.4). Additionally, when compared to their respective individual xenoestrogen responses, the xenoestrogens showed between a 41.8% - 46.5% decrease in proliferation. It's also important to consider the variability around the individual cell counts to understand the significance of the difference in Tables 4.4 and 4.5. Individual data are shown in Appendix 8. This table highlights the small variability between replicates.

Table 4.4: Summary of the % change between the combination and E2, control and the respective individual xenoestrogen tested in the combination at their maximum response (day 6). Yellow = % decrease and green = % increase. * = represented in Table 4.5.

	E2 (% change)	Control (% change)	Respective xenoestrogen (% change)
EE2 + E2	15.1	21.4	1.8
BPA + E2	7.1	16.9	2.4
Genistein + E2	47.4	31.9	43.0
Butylparaben + E2	28.8	7.8	41.8
Tetrahydrocurcumin + E2	46.6	30.8	42.7
Kaempferol + E2	54. 9	38.4	46.5
Estriol + E2	48.9	30.8	45.1
Combination	23.0	0.2	*

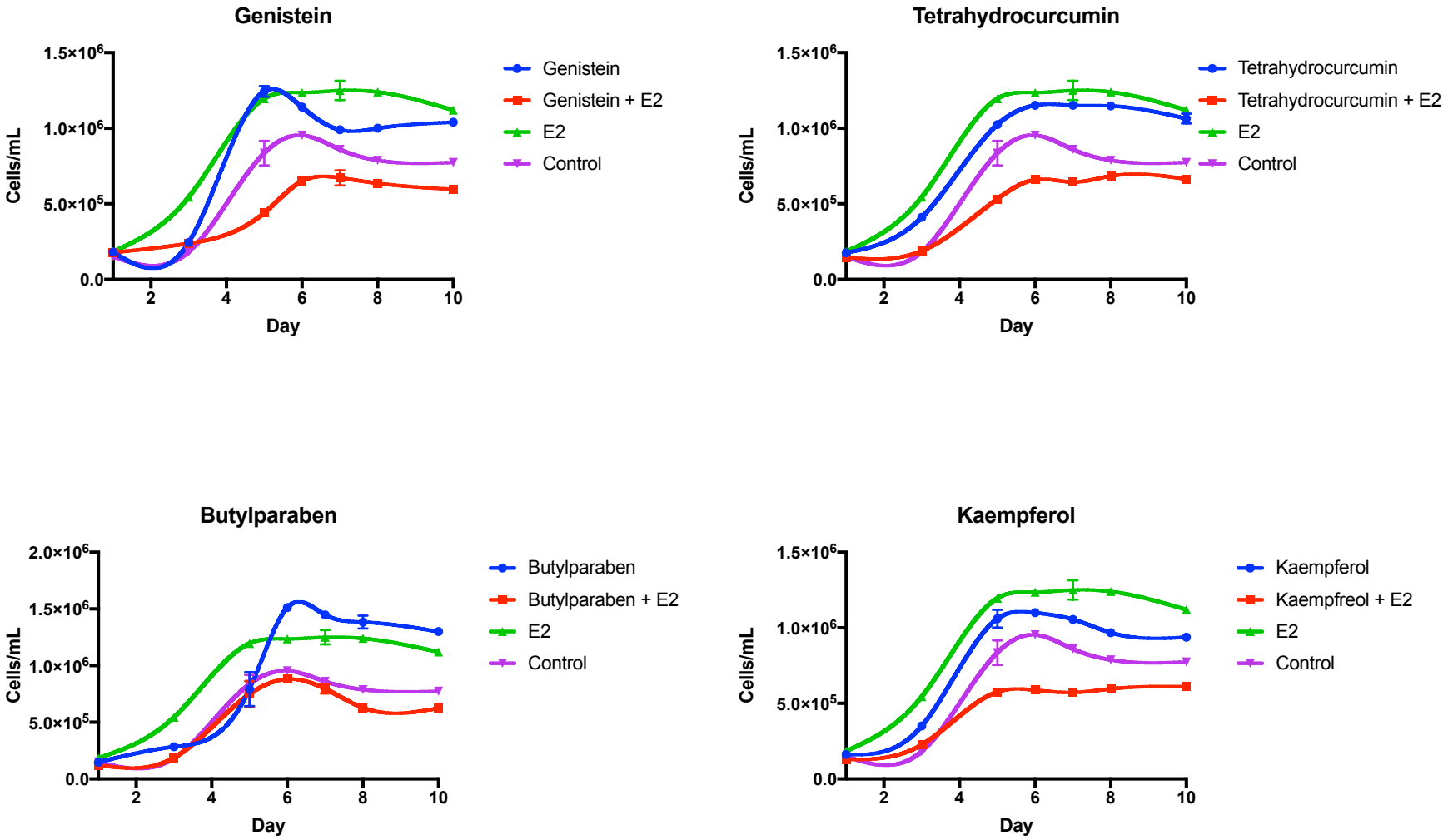


Figure 4.5: also see page 126

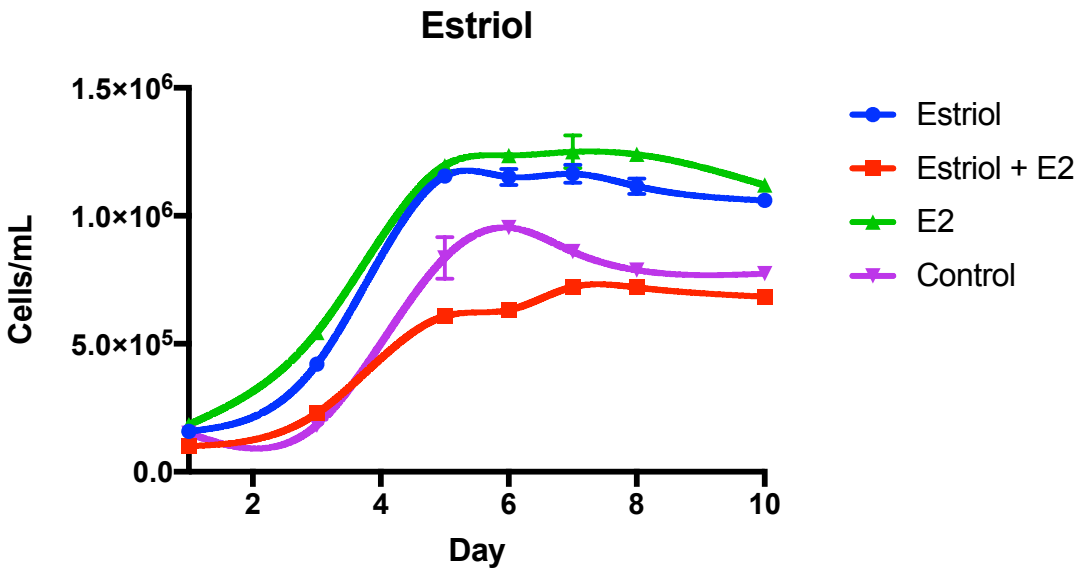


Figure 4.5 (see page 124): MCF-7 proliferation (± SEM, n=3) response to individual and combinations of genistein, butylparaben, tetrahydrocurcumin, kaempferol and estriol. All of the xenoestrogens had a negative effect on MCF-7 cell proliferation compared to E2, individual compounds and the control.

Intriguingly, the combination study which included all 8 xenoestrogens from the two combination studies, had a similar growth response to the control (Fig. 4.6). It appears that the positive effect on proliferation induced by E2, EE2 and BPA was counteracted by the negative effects of genistein, butylparaben, tetrahydrocurcumin, kaempferol and estriol. Therefore, when comparing the combination study to the control, there was only a 0.2% difference in proliferation.

Table 4.5: % Decrease of the combination study compared to individual xenestrogen responses as at day 6 when the maximum response was observed.

	% Decrease
E2	23.0
EE2	33.4
BPA	15.0
Genistein	16.5
Butylparaben	37.0
Tetrahydrocurcumin	17.4
Kaempferol	13.5
Estriol	17.4

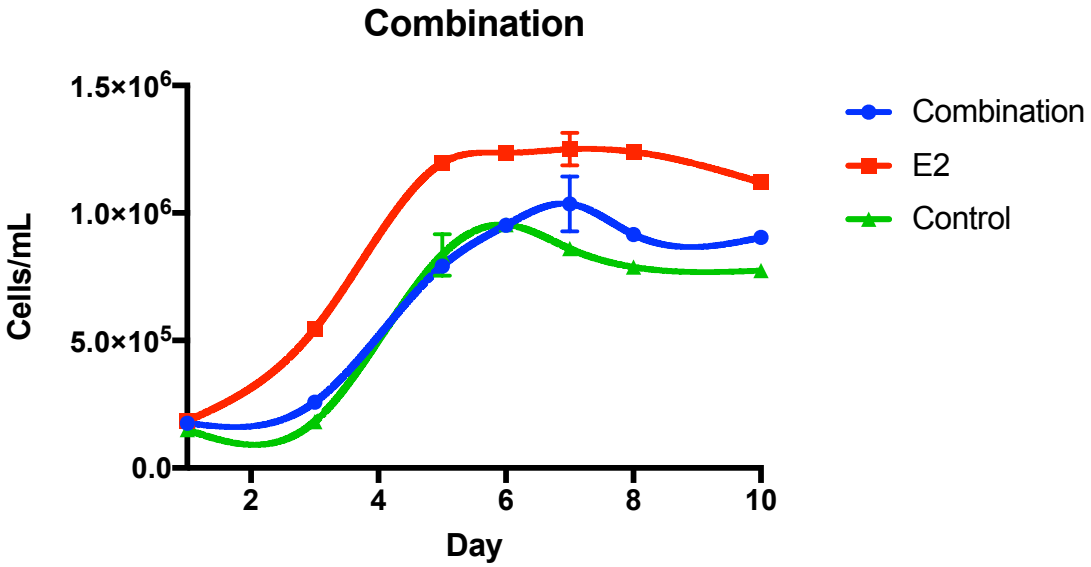


Figure 4.6: MCF-7 proliferation (± SEM, n=3) response to the combination of all eight xenoestrogens. The combination had a negative effect on proliferation compared to E2 and All of the xenoestrogens and estriol had a negative effect on MCF-7 cell proliferation compared to E2, individual compounds and the control.

Additionally, Figure 4.7 highlights the similarities between the individual xenoestrogen responses compared to the combination responses. It is clear that individually all the compounds elicit a similar proliferative effect, however, they do not behave similarly in combinations. This is illustrated by the cells' response to the combinations where a negative (ameliorative) proliferative response by the cells was observed.

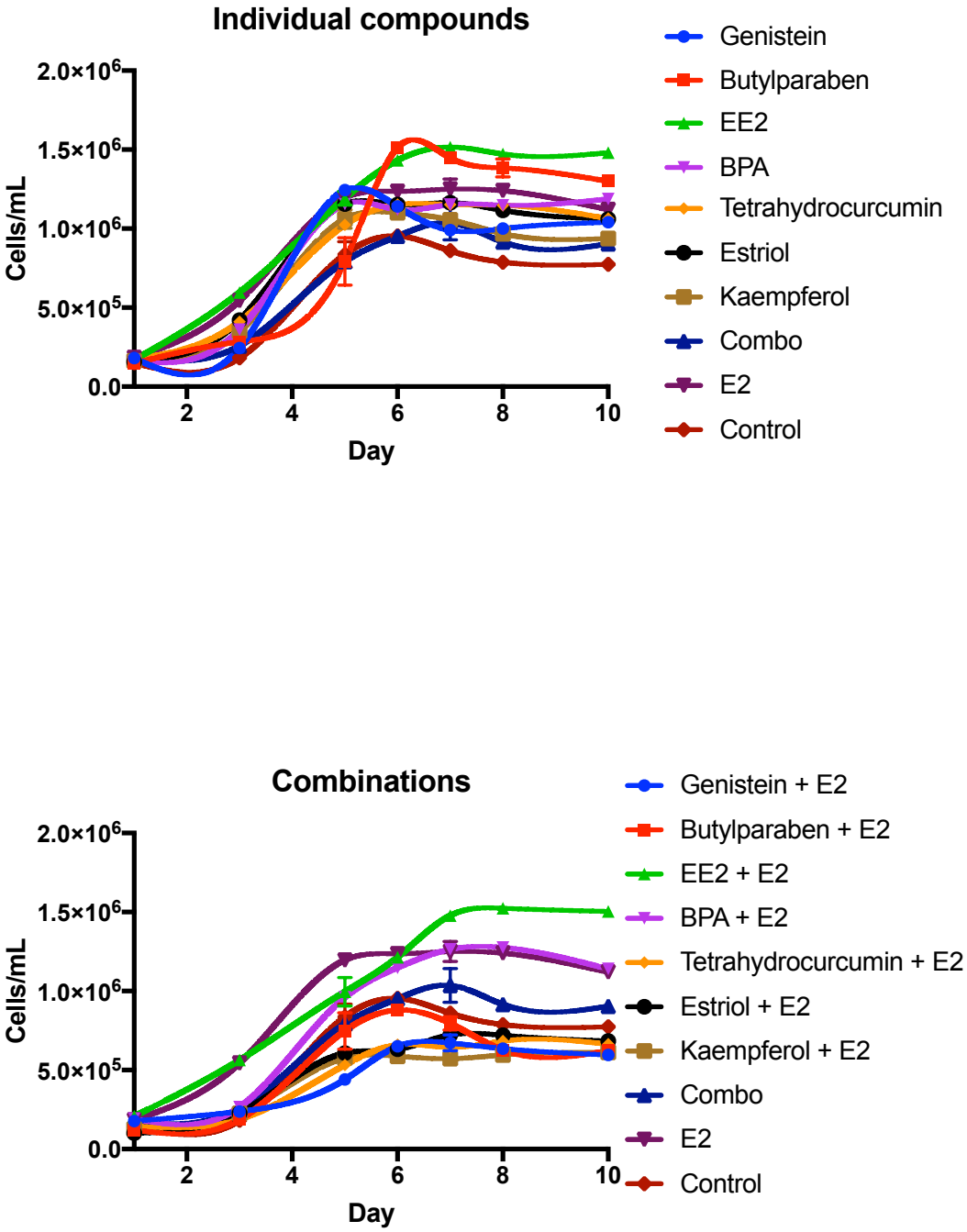


Figure 4.7: Summary of MCF-7 proliferation response (± SEM, n=3) to individual xenoestrogens and combination studies. Individually, the xenoestrogens induce a similar proliferative response at the selected concentrations. However, in combination with E2 there are clear groupings with EE2 and BPA eliciting a response akin to E2, while the remaining compound combinations elicit a response akin to or below the control. This also highlights the eight-component combination which is represented in the middle of the two combination groupings showing how the two categories of xenoestrogens can counteract each other.

4.4. Discussion

In this chapter, the estrogenic responses of individual and combinations of xenoestrogens were characterised in both dose response experiments and 10 days growth curve experiments. Due to the persistence of xenoestrogens in the environment and food, their individual estrogenicity has long been appreciated. However, a normal diet and basic daily habits result in exposure to complex mixtures of xenoestrogens. This leads to uptake, blood levels, and possibly a cellular response to these mixtures in the body. Therefore, the effects of mixtures are important for the risk assessment of xenoestrogen cocktails, particularly as possible breast cancer risk factors.

All of the xenoestrogens studied satisfy the basic criteria used to define potential estrogenicity, yet they exhibited differences in both the dose response experiments and in the 10-day growth curve experiments, when in combination with other xenoestrogens. In terms of their individual responses, MCF-7 cell proliferation profiles were as expected. However, when the cells were challenged with different combinations of xenoestrogens, whether by dose response experiments or growth curve experiments, two distinct proliferative responses were observed – those that had a positive effect on proliferation and those that had a negative effect. When comparing the 2D structures of the xenoestrogens that had a negative effect on MCF-7 proliferation, all except estriol, had at least one keto group in their structure. Interestingly, this is characteristic of many phytoestrogens which have been shown to have anti-proliferative effects on breast cancer cells, such as MCF-7. Butylparaben in combination with E2 was not expected to have a negative effect on proliferation; however, when one considers the high degree of structural similarity between butylparaben and genistein (Fig. 4.8) it is obvious that butylparaben could have the same effect as genistein on purely structural grounds. This is highlighted by the positioning of the hydroxyl groups and keto groups when overlaid with genistein (Figure 4.8).

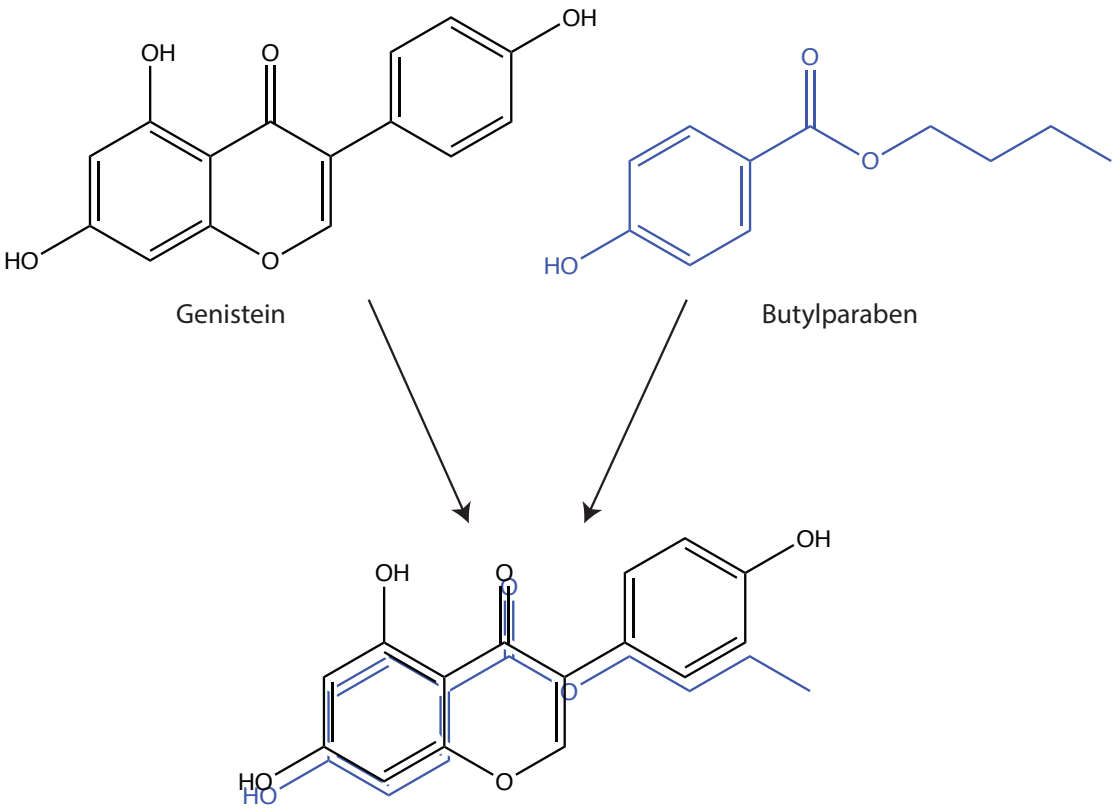


Figure 4.8: Structural comparison between genistein (black) and butylparaben (blue), highlighting the clear similarities showing the structural alignment of keto and hydroxyl moieties.

Previous studies have shown that phytoestrogens have anti-proliferative effects in breast cancer cell model systems. Genistein is a classic example; it has been shown to positively affect proliferation at low concentrations (e.g. nM) and negatively affect proliferation at high concentrations (e.g. µM) (Lavigne*, et al.*, 2008, Lecomte*, et al.*, 2017). Epidemiological studies have also shown genistein to be protective against breast cancer in women of child-bearing age (who have high circulating E2 levels) but not for postmenopausal women (who have low circulating E2 levels) (Adlercreutz, 1995, Messina*, et al.*, 2006). In this study, genistein had anti-proliferative effects on MCF-7 cells at lower (e.g. nM) concentrations in conjunction with E2, compared to individual studies which saw the anti-proliferative effect at high concentrations (e.g. µM). This is the case for the other anti-proliferative xenoestrogens, which had a positive effect on proliferation individually but a negative effect in combination with E2. However, when you consider the concept of simple additivity, where xenoestrogens act via the ER's LBC, one would not expect there to be different proliferation responses because they all bind to the one site, LBC. It is clear that mixtures of xenoestrogens behave differently depending on the structures of the individual components of the cocktail.

The phytoestrogens are the most studied group of xenoestrogens that have anti-proliferative effects on MCF-7 cells, and multiple mechanisms of action have been proposed. Firstly, of all the compounds that were shown to have negative proliferative effects in the MCF-7 cell studies, almost all of them have been shown to have an ERβ preference (Morito*, et al.*, 2001). It is well documented that ERβ has modulatory effects on ERα-mediated signalling, thus, this could logically explain the anti-proliferative effects (Matthews*, et al.*, 2006, Zhou*, et al.*, 2001); however, MCF-7 cells express very low levels of ERβ compared to ERα; thus, it is unlikely that the low level of ERβ expression would be enough to cause the 40-50% decrease in proliferation, which was observed in these studies. In addition, Tetrahydrocurcumin has not been extensively studied in the context of ER binding; therefore, it is the only compound where an ER isoform preference is not known.

Another possible mechanism that could explain the decrease in cell proliferation is direct competition for ERα LBC. However, binding at the LBC is not simply a case of binding, it involves an equilibrium between bound (e.g. hydrogen bonds with LBC amino acid residues) or an unbound state (Fig. 4.9). This equilibrium is displaced towards the bound state if the ligand has a higher binding affinity for the LBC. For example, E2 binds with high affinity to the LBC, which leads to an equilibrium shift towards the bound state. However, for a ligand that has a lower binding affinity (e.g. genistein) the equilibrium will favour the unbound state in comparison to E2. Therefore, depending on individual ligand concentrations, lower LBC affinity xenoestrogens can outcompete E2, overwhelming the LBC and leading to an overall negative effect on proliferation. However, when you compare the combination proliferation response to the individual xenoestrogen proliferation response, you would not expect to see a 40-50% decrease with such high theoretical LBC occupancy. Therefore, direct competition at the LBC is not likely to be the mechanism responsible for the negative proliferative effects.

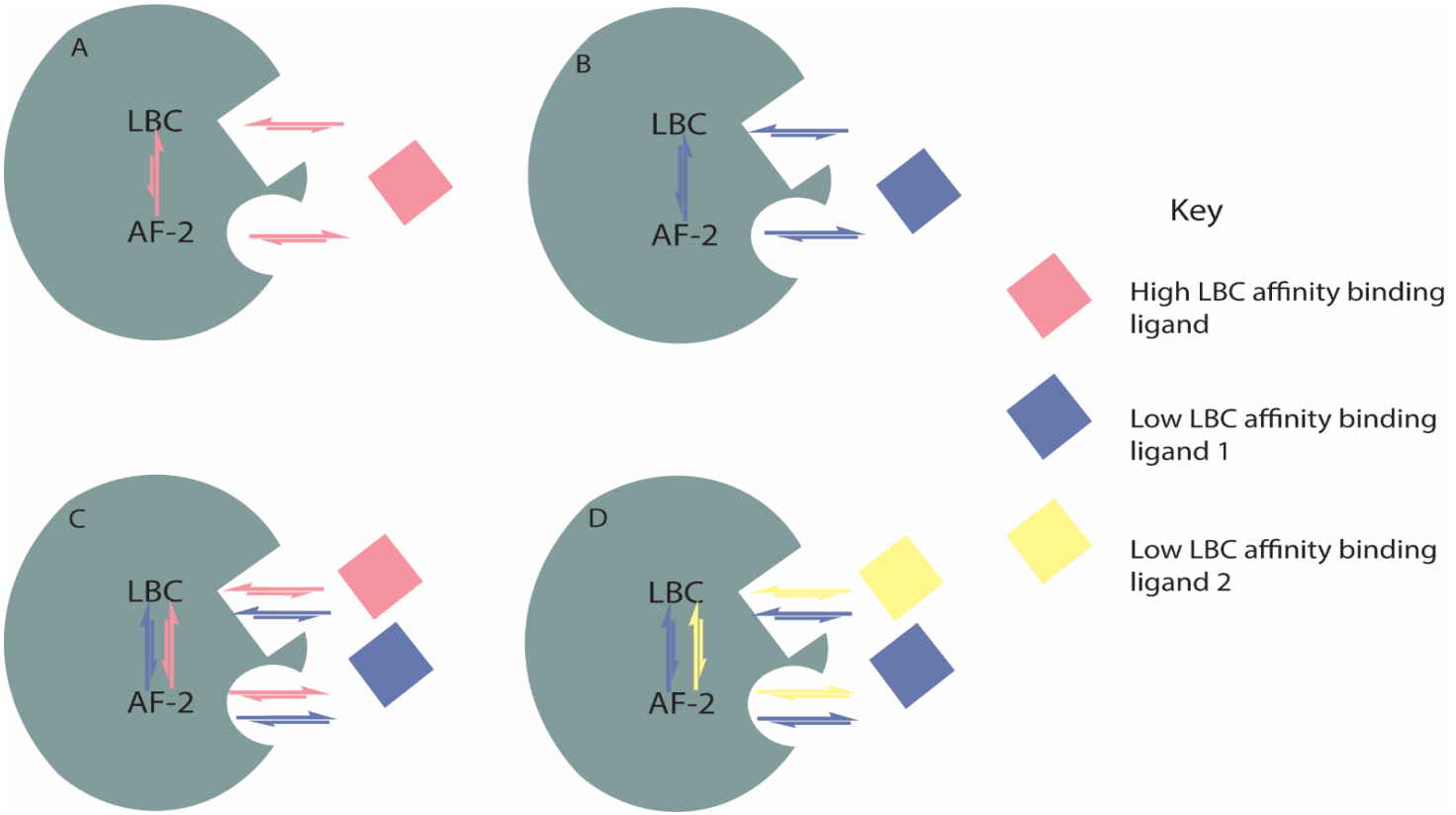


Figure 4.9: Illustration of equilibria involved in ER/ligand interactions. A) shows the binding equilibria for a high LBC affinity binding ligand in which binding to the LBC is favoured over AF-2. B) shows the binding equilibria for a low LBC affinity binding ligand in which the binding affinities for LBC and AF-2 are similar, thus LBC binding affinity is favoured less compared to the high LBC affinity binding ligand. C and D both show the complex binding equilibria for combinations. C shows a combination with a high and low LBC binding affinity ligand which shows that the high LBC affinity binding ligand will outcompete the low LBC binding affinity ligand for the LBC binding site, facilitating AF-2 spill over. On the other hand, D shows that both ligands have similar binding affinities for both binding sites, therefore a high concentration of at least one ligand is going to be required to facilitate AF-2 spill over.

Another possible explanation for the mixed proliferative/anti-proliferative effects is akin to that previously described for 4-OHT's mechanism of action (Jensen, 2001, Jensen*, et al.*, 2004). 4-OHT also shows the same mixed proliferative/anti-proliferative effects observed in my studies, where at low concentrations it acts as an agonist and this positively affects MCF-7 cell proliferation, whereas at high concentrations it acts as an antagonist, negatively affecting MCF-7 cell proliferation. A two-site model has been proposed to explain 4-OHT's actions on proliferation (Jensen*, et al.*, 2004, Wang*, et al.*, 2006). In this model it is suggested that a primary, high affinity site is responsible for the agonist activity and a secondary, low affinity site is responsible for the antagonist activity (Wang*, et al.*, 2006). In addition, Wang and colleagues (2006) carried out structural investigations which support the two-site model hypothesis; they showed that 4-OHT binds classically to the LBC and a second 4-OHT molecule binds to the AF-2 site, a hydrophobic groove in ERβ. While this 4-OHT study focuses on ERβ, analysis of amino acid residues involved in the interaction of 4-OHT at AF-2 indicates that the amino acids are conserved between the two ER isoforms, suggesting ERα may also have the capacity to interact with more than one small, non-peptide molecule rather than the normal peptides of co-activator proteins that are normally used to study the AF-2. Assuming the ER translocates to the nucleus when bound by two 4-OHT molecules, binding at the AF-2 site is likely to significantly affect the recruitment of coactivator proteins and the formation of the coactivator complex. Coactivators have specific enzyme activity (see Section 1.3.1.2) which leads to ER-mediated transcription; therefore, this could be disrupted by AF-2 binding, blocking histone modifications, or preventing rapid degradation by the proteasome. Ligand binding rapidly signals ER ubiquitylation, and ubiquitylated ER cycles on and off the ERE promoter site to control target gene transcription. Emerging evidence suggests that interactions between genomic and non-genomic signalling pathways mediated ER ubiquitylation. For example, SRC-mediated phosphorylation at Tyr537 (ERα) primes ER E6AP recruitment. E6AP is an E3 ligase, a protein that recruits a ubiquitin-conjugating enzyme which assists in the transfer of ubiquitin to the ER and is recruited as part of the coactivator complex. The ubiquitylated ER is then signalled to the proteasome for degradation (Zhou*, et al.*, 2014). Therefore, disruption of this ubiquitylation and AF-2 occupancy by 4-OHT could prevent key protein recruitment which are involved in catalysing the 'opening' chromatin, rendering the ER promoter site 'unavailable' to transcriptional machinery.

This would likely downregulate ER-mediated transcription and reduced cell proliferation (Fig. 4.10).

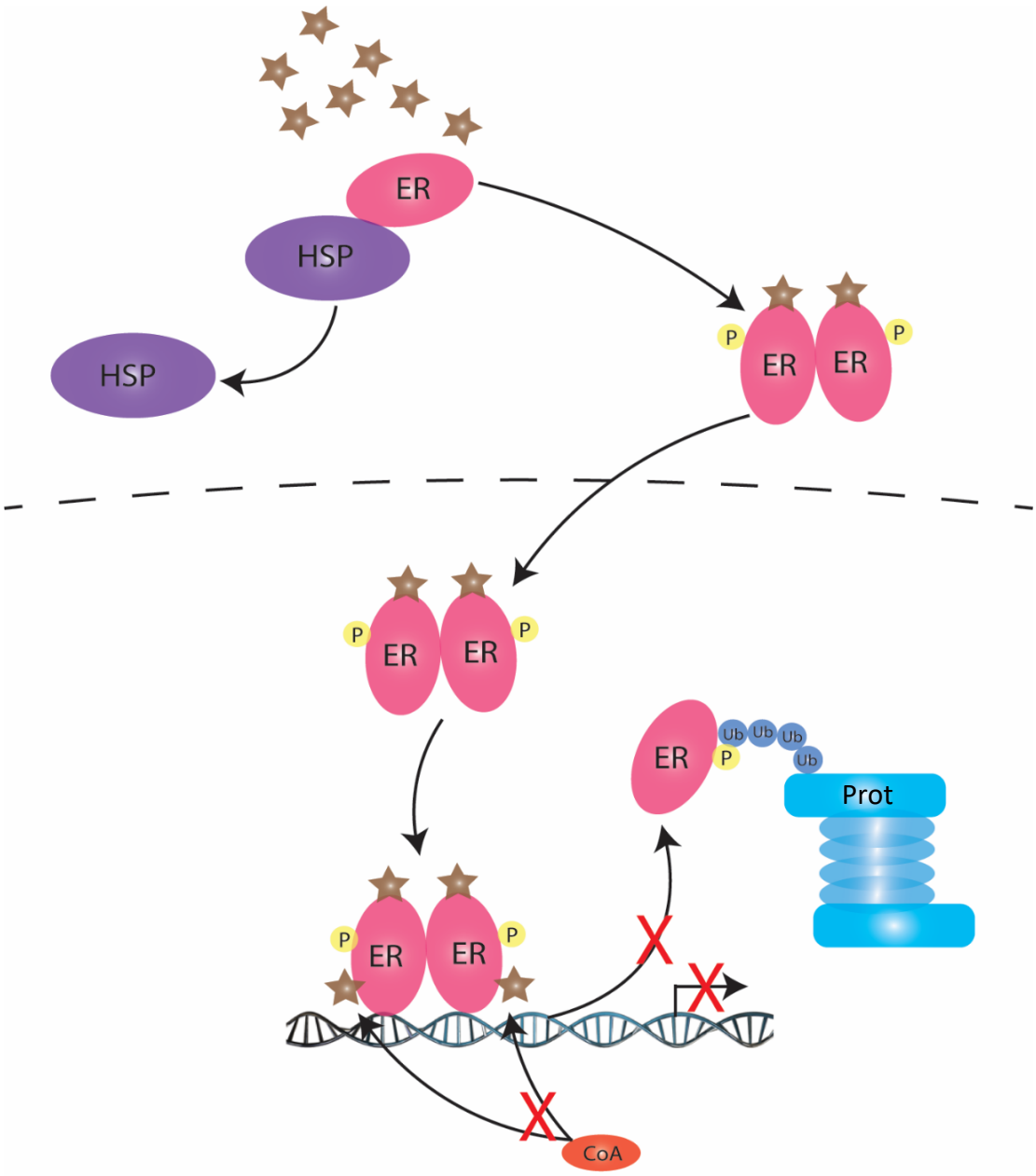


Figure 4.10: Illustration of the 4-hydroxytamoxifen (4-OHT) two site binding model and the potential downstream implications on ER-mediated transcription and proteolysis. The ER monomer is activated upon 4-OHT binding, facilitating dimerisation and translocation to the nucleus. The ER dimer binds to the estrogen response element (ERE) on the DNA but the 4-OHT/AF-2 interaction disrupts coactivator (CoA) recruitment and ER degradation at the proteasome (Prot) leading to altered gene transcription. P=phosphate and Ub=ubiquitin (from Zhou *et al.,* 2014 with permission).

It is tempting to speculate that the proposed two-site model for 4-OHT could explain the proliferation responses seen in my studies, both on individual compounds and in mixtures. This study shows a number of xenoestrogens having the same proliferative response as seen in the previously reported 4-OHT study; therefore, the anti-proliferative effect could be caused by an interaction of some xenoestrogens at AF-2. It has been suggested that the AF-2 site is the secondary, low affinity site which aligns with the experimental results presented here. If the LBC is the primary, high affinity site then logically it would need to be fully occupied before any compound would bind to the second, low affinity site (i.e. AF-2). This could explain the high xenoestrogen concentration dependence before any anti-proliferative effects are seen. However, when E2 is added to the mixture, the concentration of xenoestrogen required to have a negative anti-proliferative effect decreases. Since E2 has a high affinity for the LBC, it would, in theory, compete with the xenoestrogen in the mixture for LBC binding, reducing the required amount of xenoestrogen to reach full LBC occupancy. This would facilitate AF-2 spill over at lower concentrations. In a manner akin to the proposed 4-OHT two-site model, the same outcomes would be expected for xenoestorgen ER-mediated actions in MCF-7 cells (see Fig. 4.11).

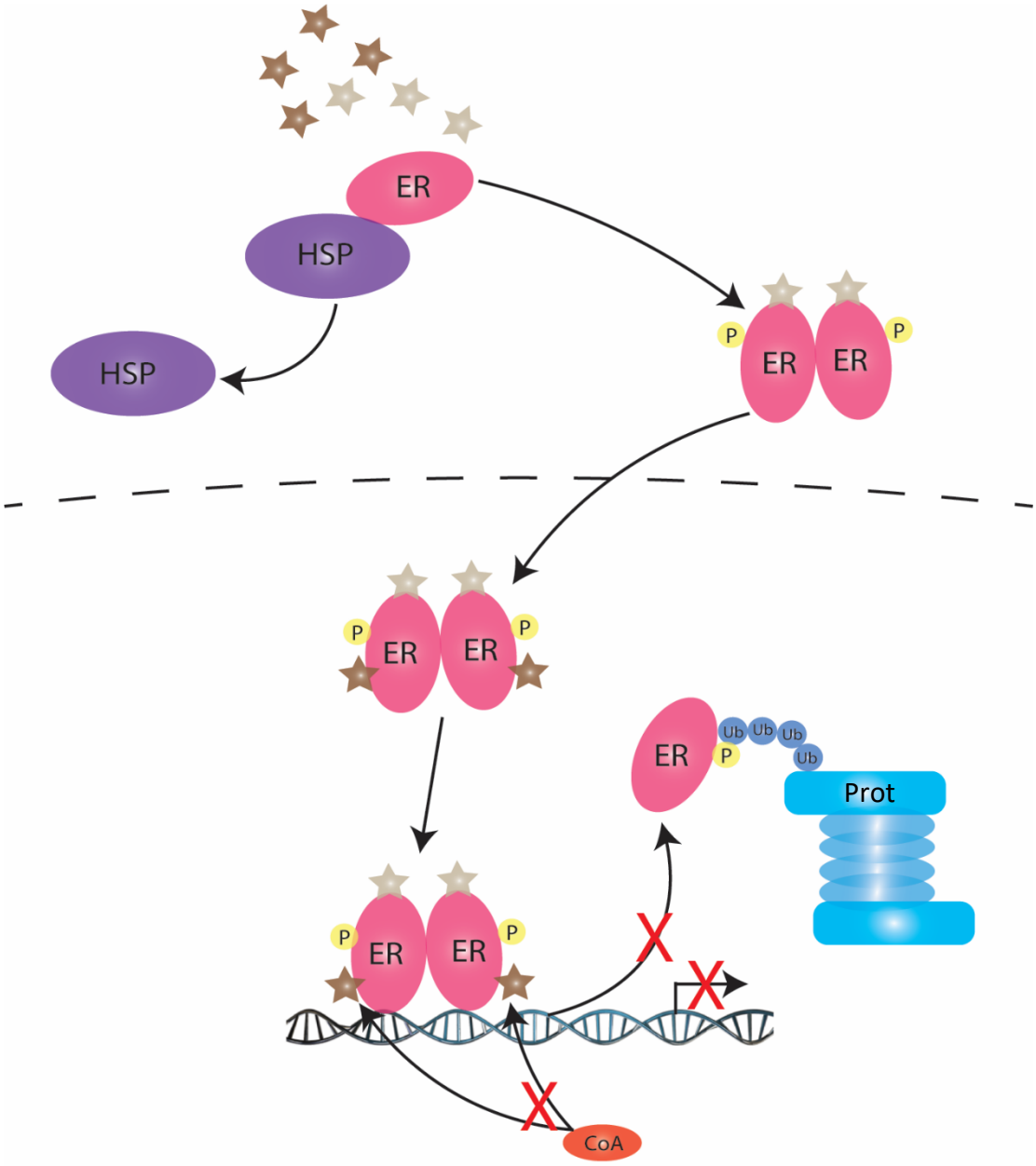


Figure 4.11: Illustration of the xenoestrogen two site binding model and the potential downstream implications on ER-mediated transcription and proteolysis. The ER monomer is activated upon E2 binding, facilitating dimeristaion and translocation to the nucleus. The ER dimer binds to the estrogen response element (ERE) on the DNA but the xenoestrogen/AF-2 interaction disrupts coactivator (CoA) recruitment and ER degradation at the proteasome (Prot) leading to altered gene transcription. P=phosphate and Ub=ubiquitin (from Zhou *et al*., 2014 with permission).

The studies reported here show that the xenoestrogens studied can be categorised by their responses in dose response and 10-day growth curve experiments. A majority of the xenoestrogens selected were chosen due to their previously reported mixed agonist/antagonist responses and their persistence in the environment and in food, which meant that they were akin to daily human exposures. These studies confirmed and further support the hypothesis that some xenoestrogens have a concentration-dependent anti-proliferative effect, which is amplified in the presence of E2. This appears to be the first time butylparaben has been reported to have a mixed agonist/antagonist effect in MCF-7 cells. However, when comparing xenoestrogen structures, it is clear that butylparaben has a high structural analogy to genistein and other flavonoids and therefore, it is not surprising to find a similar proliferation prolife for butylparaben.

Interestingly, the combination study, which incorporated eight different xenoestrogens, had the same effect on proliferation as the control, however, this combination study exhibited a 22.97% decrease when compared to E2 proliferation. For the first time, a two-site model has been suggested to explain the agonist/antagonist activity exhibited by genistein, kaempferol, tetrahydrocurcumin, butylparaben and estriol, which display the same mixed proliferation response as the breast cancer drug metabolite, 4-OHT. Therefore, if xenoestrogens do function via a two-site model, it could have a profound pharmacological significance in the search for a drug to prevent breast cancer. This will be investigated further in Chapters 5 and 6.

4.5. Concluding Remarks

The results from this chapter clearly show the MCF-7 proliferative response to the xenoestrogen combination, with two clear categories of xenoestrogens emerging. EE2 and BPA clearly had an additive effect on MCF-7 cell proliferation, while genistein, butylparaben, tetrahydrocurcumin, kaempferol and estriol all had anti-proliferative effects on MCF-7 cells. In my opinion, these effects are likely ERα-mediated since MCF-7 cells are known to express high levels of ERα which facilitates cell proliferation. Therefore, it is possible that the xenoestrogens that had anti-proliferative effects on MCF-7 cells are binding the AF-2 site (a secondary, low affinity binding site) on the ER. This two-site binding model has previously been proposed to explain

the mixed agonist/antagonist effect of 4-OHT, thus it is plausible that xenoestrogens are behaving in a similar manner. However, it is also conceivable that other mechanisms not involving ERα are also involved in facilitating the anti-proliferative effects. In order to study this, experiments were performed using ERα and ERβ CALUX® assays to investigate the ER-mediated responses of xenoestrogen combinations.

Chapter 5 CALUX® Exposure Studies

5.1. Introduction

There has been considerable effort over the last two decades to develop *in* vitro assays capable of screening the estrogenic activity of environmental pollutants, industrial chemicals, natural chemicals and pharmaceuticals (Charles, 2004, Mueller, 2002, Mueller, 2004, Scrimshaw*, et al.*, 2004, Soto*, et al.*, 2006, Zacharewski, 1997). While many studies have identified effects emanating from individual xenoestrogens, there is scientific evidence that suggests mixtures of xenoestrogens will predominantly elicit additive effects because they bind to the ERs *in vitro* as well as *in vivo*. Individual xenoestrogens are usually present in low concentrations, mostly below their individual maximum detected levels, but have been shown to act additively, thereby eliciting an effect, even when applied in combination with the individual compounds at concentrations below their No Observed Adverse Effect Level (NOAELs). Interestingly, most of these studies have analysed individual and combination effects of xenoestrogens at ERα, while few have dealt with effects of individual and combinations of xenoestrogens at ERβ, reviewed by (Gustafsson, 1999). However, the ERα and ERβ subtypes can interact with a wide variety of different xenoestrogens, with some exhibiting different relative affinities for the ER subtypes. Therefore, the expression of ER isoforms, and the cells and tissues in which they are expressed, are important for understanding estrogen-mediated cellular functions (Lee*, et al.*, 2012). Thus, the intracellular ratio of the two receptors is important in understanding xenoestrogen combinations as breast cancer risk factors. This will be discussed below in section 5.1.3.

5.1.1. Genomic and Non-Genomic Mechanisms of ER Binding

E2 determines its effects by binding to ERα or ERβ which act as ligand transcription factors regulating the transcription of ERE-containing genes. In addition, E2 is also able to elicit the rapid activation of extra-nuclear signalling pathways via interactions with membrane-localised receptors. Integration of nuclear and extra-nuclear ER-dependent actions as well as of ERα and ERβ specific signalling co-ordinately contributes to the regulation of the E2 physiological actions (Ascenzi*, et al.*, 2006, Marino*, et al.*, 2012). All the E2 effects occur in parallel with transcriptional and post-translational modulation of ER intracellular concentrations, which are finely modulated by E2-induced extra-nuclear (Caiazza*, et al.*, 2007, La Rosa*, et al.*, 2012) and epigenetic signalling (e.g. ER promoter methylation) (Thomas*, et al.*, 2011). For example, the relative concentrations of ERα and ERβ are significantly altered during the development of breast cancer with an increase in ERα levels and a decrease in ERβ concentration (Roger*, et al.*, 2001). In addition, E2 protective effects against colon cancer growth rely on E2-induced ERβ up-regulation. In addition, ERα degradation is also required for the transcription of E2 responsive genes (Caiazza*, et al.*, 2007). As a whole, this evidence points to the control of ERα as a critical step in endocrine-dependent cell growth and consequently, the identification of molecules that modulate these molecular circuities is a demanding issue(Leclercq*, et al.*, 2006, Reid*, et al.*, 2003).

Xenoestrogens are heterogeneous chemicals known to bind to ERα and ERβ, which can interfere with many aspects of estrogen-dependent control of body homeostasis including the balance between cell growth/apoptosis (Diamanti-Kandarakis*, et al.*, 2009, Zoeller*, et al.*, 2012). Although xenoestrogens act via the same binding site (i.e. LBC), they interfere with ER-mediated signalling, driving breast cancer cells to different functional outcomes (Bolli*, et al.*, 2008, Bulzomi*, et al.*, 2010, Marino*, et al.*, 2012). In particular, some xenoestrogens (e.g. genistein) cause proliferative effects in breast cancer cells at nanomolar concentrations, but anti-proliferative effects at micromolar concentrations, and have even been shown to have protective effects against breast cancer in women of child bearing age (see Section 1.4.1.) (Chang*, et*

al., 2008). Therefore, understanding the individual ER isoform effects is important in understanding xenoestrogens as breast cancer risk factors.

5.1.2. ERα and ERβ Ratio in Humans

While estrogens and ERs are primarily associated with female reproductive development, the role of ERs in the body is often overlooked. The ER's expression patterns and functions vary in receptor subtype and in cell- and tissue-specific manners, leading to growth and differentiation in both males and females. ERs are expressed early in fetal development, with trophoblast cells (i.e. cells that eventually develop into the placenta) being the first cells during embryonic development to have aromatase activity, indicative of estrogen production (see Fig. 1.3) (Stocco, 2012, Vasquez*, et al.*, 2013). At 9 weeks, the placenta becomes the primary source of fetal estrogen production and the embryonic sex organs begin to differentiate. Interestingly, the expression of the ERs, in particular ERβ, during development is not limited to reproductive development, indicating the importance of ERβ dependent E2 signalling in the fetus (Berkane*, et al.*, 2017, Devroey*, et al.*, 1990). However, aberrant estrogen signalling, caused by xenoestrogens, can lead to birth defects in both males and females. Indeed, the administration of DES to pregnant women in the 1950s led to increased risk of reproductive tract development abnormalities in both male and female babies (Hoover*, et al.*, 2011). Interestingly, while breast cancer is often thought of as a gender-related cancer, breast cancer cells respond to estrogens in a growth and development context, similar to that observed in the fetus, rather than as purely a female reproductive response (Gillies*, et al.*, 2010, Heldring*, et al.*, 2007). Therefore, considering breast cancer cells as primordial cells rather than as breast cells could help understand disease risk.

5.1.3. ERα and ERβ Ratio in the Breast

In general, the ERα isoform expression occurs mainly in tissues related to reproductive activity (e.g. uterus and mammary gland), ERβ is more widely distributed (see Section 1.1.). Conversely, in the clinical setting ERα is the primary focus with approximately 75% of diagnosed breast cancers being identified as ERα positive tumours (Lim*, et al.*, 2016); however, the ERβ isoform is often overlooked. Contrary to the clinical setting of breast cancer, ERβ is highly expressed in the normal breast epithelium and declines during breast tumourgenesis (i.e. in precancerous and

cancerous lesions) (Abdel-Fatah, *et al.*, 2008, Chantzi, *et al.*, 2014, Hung, 2004, Jarvinen, *et al.*, 2000, Roger, *et al.*, 2001, Shaaban, *et al.*, 2003, Speirs, *et al.*, 2002). Interestingly, higher levels of ERβ expression in breast tumours have been found to be associated with the expression of good prognostic markers or better clinical outcomes (Guo, *et al.*, 2014, Haldosen, *et al.*, 2014, Kim, *et al.*, 2012). Clearly the intracellular ratio between ERα and ERβ is important during breast cancer development. ERβ is also regarded as a negative regulator of ERα (Bottner, *et al.*, 2014); thus, the risk emanating from an estrogenic substance depends on its activity at both ER subtypes. This emphasises the importance of identifying the ability of xenoestrogens (individual and combinations) to activate ERα and ERβ-mediated transcription in the cumulative risk assessment of xenoestrogens (Seeger, *et al.*, 2016). This will be especially important for xenoestrogens such as genistein, which have a binding affinity preference for ERβ over ERα.

The aim of this study was to understand the importance of the intracellular ratio of the two receptors ERα and ERβ for the xenoestrogens: E2, EE2, genistein, BPA, methylparaben and butylparaben. To this end, the activity of individual and combinations of xenoestrogens to activate either ERα or ERβ-mediated transcription was characterised and compared to that of E2, using the ERα and ERβ CALUX® assay.

5.2. Experimental Approach

ERα and ERβ CALUX® cell lines were kindly provided by Biodetection Systems (Amsterdam, The Netherlands). Each cell line had been genetically modified to express the ER isoform (α or β) along with a luciferase reporter gene as described earlier (Section 2.2.9.2.).

5.2.1. ERα and ERβ CALUX® Assay

The ERα and ERβ CALUX® assays were performed on individual compounds to evaluate the individual effects of each xenoestrogen on ERα and ERβ. Full dose response (13 increasing concentrations) curves were tested for each compound and the EC_{50} values were determined (see Section 2.2.9.2). The calculated EC_{50} values were applied to predict the responses of mixtures using the well-known concept of

CA, based on the work of Loewe and Muischnek (Loewe*, et al.*, 1926). CA applies the concept of pure additivity, thus if a correlation was observed between experimental and the predicted CA responses, additivity was assumed (Kortenkamp, 2007). In addition, xenoestrogen mixtures (Table 5.1) were studied in both ERα and ERβ CALUX® assays (see Section 2.2.9.2.). The mixtures were designed to include a full dose-response (13 increasing concentrations) of one xenoestrogen (varied) with a fixed concentration of either one or two additional xenoestrogens (see Tables 5.1, 5.2 and 5.3). The concentrations selected for both the varied xenoestrogen and fixed xenoestrogens were based on initial experimental data from individual response curves, with the fixed concentrations selected based on EC_{10} values (Table 5.2).

Table 5.1: Xenoestrogen combinations studied in both ERα and ERβ CALUX® assays.

	Fixed ▾							
Varied ▾	E2	EE2	Genistein	BPA	Methylparaben	Butylparaben	Combination 1	Combination 2
E2		✓	✓	✓	✓	✓	✓	✓
EE2	✓		✓	✓	✓	✓	✓	✓
Genistein	✓	✓		✓	✓	✓	✓	✓
BPA	✓	✓	✓		✓	✓	✓	✓
Methylparaben	✓	✓	✓	✓		✓	✓	✓
Butylparaben	✓	✓	✓	✓	✓		✓	✓

Table 5.2: Fixed concentrations of xenoestrogens/E2 added to each combination in the ERα and ERβ CALUX® assays.

Xenoestrogen	ERα	ERβ
E2 (M)	3.0×10^{-12}	2.0×10^{-10}
EE2 (M)	3.0×10^{-13}	7.0×10^{-10}
Genistein (M)	3.0×10^{-8}	6.0×10^{-10}
BPA (M)	1.0×10^{-7}	1.0×10^{-7}
Methylparaben (M)	3.0×10^{-7}	1.0×10^{-5}
Butylparaben (M)	3.0×10^{-8}	3.0×10^{-7}

Table 5.3: Compositions and concentrations of Combinations 1 and 2.

		BPA	Genistein	Methylparaben	Butylparaben
Combination 1	ERα	1.0×10^{-7}	3.0×10^{-8}		
	ERβ	1.0×10^{-7}	3.0×10^{-10}		
Combination 2	ERα			1.0×10^{-7}	2.2×10^{-8}
	ERβ			1.0×10^{-5}	3.0×10^{-7}

5.2.2. Data Analysis

The solvent controls were subtracted from the raw luminescence values and normalised to the response of E2 in the respective assay (e.g. ERα or ERβ). The data were then plotted using non-linear regression in the software package Graphpad Prism, and EC_{50} values were calculated.

5.2.3. Cytotoxicity Testing

Cytotoxicity was studied to ensure the responses elicited by the xenoestrogens tested were not a result of cytotoxicity, but rather ERα and ERβ-mediated effects (Section 2.2.9.3.). The cytotox CALUX® assay consists of a general genetic modification of U2OS cells (same as ERα and ERβ CALUX® cells) to constitutively express a high level of luciferase. The luciferase response is not directly related to a specific pathway but serves as measure of cytotoxicity and as a control for non-specific activation or inhibition of luciferase expression or activity. If cells are exposed to cytotoxic compounds the resulting amount of luciferase expressed will decrease (van der Linden, *et al.*, 2014). All individual xenoestrogens were tested, along with combination 1 (ERα) and combination 2 (ERα and ERβ) (Table 5.3). Three

component combinations were selected based on the likelihood that cytotoxicity would be observed at higher total xenoestrogen concentrations.

5.3. Results

The estrogenic potencies of individual and combinations of xenoestrogens were measured using the ERα and ERβ CALUX® assays, and compared to the activity of the natural ligand, E2. Treatment of both cell lines resulted in mixed responses, with a wide range of maximal luciferase responses being observed for both individual xenoestrogens and mixtures. Interestingly, genistein and butylparaben induced a luciferase response that exceeded the maximum response elicited by E2; additionally, a number of combinations also exhibited this response, known as a supramaximal effect.

5.3.1. Dose-response Analysis of Individual Xenoestrogens

Concentration response curves for the individual xenoestrogens are shown for ERα and ERβ CALUX® assays in Fig. 5.1. It shows that the xenoestrogens tested in the ERα CALUX® assay exhibited a wide range of potencies, with EC_{50} values ranging from picomolar to high micromolar concentrations (Table 5.4). It was shown that the most potent xenoestrogens tested were EE2 and E2 and the least potent was methylparaben. Fig. 5.1 shows that the xenoestrogens tested in the ERβ CALUX® assay exhibited a narrower range of potencies compared to the ERα CALUX® assay, with EC_{50} values ranging from nM to high µM concentrations. As in the ERα CALUX® assay, EE2 and E2 were the most potent xenoestrogens tested, with methylparaben being the least potent. Additionally, genistein also had a similar potency to E2 and EE2, which was not observed in the ERα CALUX® assay. Supramaximal responses (responses greater than the maximal response for the natural ligand E2) were observed in both the ERα and ERβ CALUX® assay. Genistein, 123.4% and 197.0%, and butylparaben 185.3% and 176.0% both had supramaximal luciferase responses in the ERα and ERβ CALUX® assays, respectively. In addition, BPA also exhibited a supramaximal luciferase response of 127.3% in the ERα CALUX® assay but not in the ERβ CALUX® assay. Clearly, the supramaximal effect

varied between both assays and xenoestrogens with responses up to almost 200% compared to maximal luciferase response of E2.

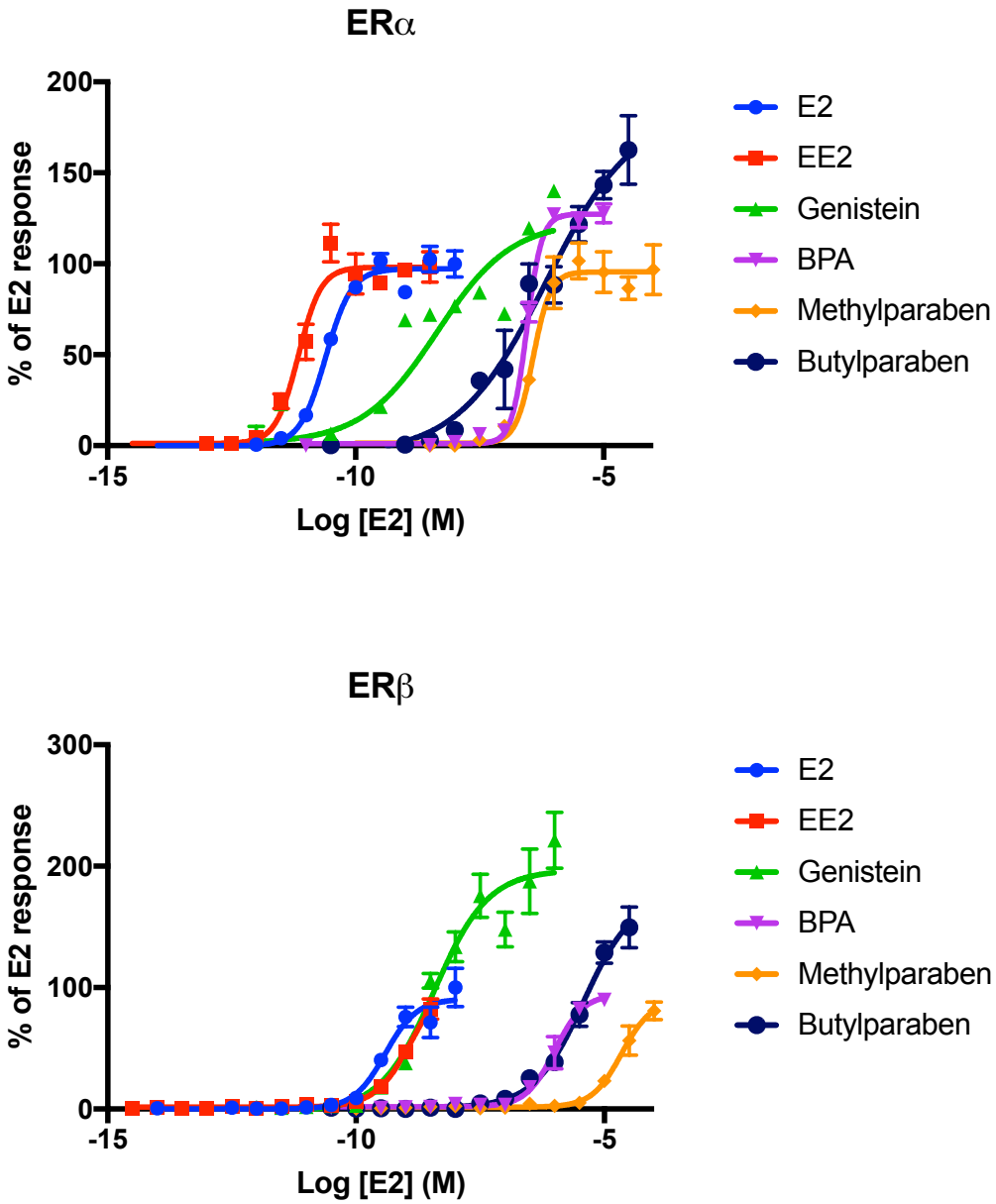


Figure 5.1: Graphs show the results of individual xenoestrogen responses in the ERα and ERβ CALUX® assays. Each individual xenoestrogen was tested in triplicate and results are presented as a non-linear regression with errors expressed as SEM.

Table 5.4: Experimental EC50 values and maximal luciferase responses for individual xenoestrogens in the ERα and ERβ CALUX® assay.

	ERα		ERβ	
	EC_{50} (M)	Maximal response (%)	EC_{50} (M)	Maximal response (%)
E2	2.5×10^{-11}	100.0	3.9×10^{-10}	90.7
EE2	7.1×10^{-12}	98.1	1.3×10^{-9}	111.5
Genistein	4.4×10^{-9}	123.4	3.8×10^{-9}	197.0
BPA	2.8×10^{-7}	127.3	1.0×10^{-6}	94.5
Methylparaben	3.8×10^{-7}	95.5	2.2×10^{-5}	89.7
Butylparaben	7.0×10^{-7}	185.3	3.8×10^{-6}	176.0

5.3.2. Dose-Response Analysis of Xenoestrogen Combinations

Combinations of different xenoestrogens exhibited a wide range of potencies, depending on the xenoestrogen combination and ER subtype tested (see Tables 5.5 and 5.6). In addition, the maximal responses of the combinations varied depending on the combination of the xenoestrogens tested. This is summarised in Tables 5.7 and 5.8.

Table 5.5: Comparison of experimental (E) and CA predicted (P) ERα CALUX® EC_{50} values for the xenoestrogen combinations. * indicates a response was not converged using non-linear regression.

Fixed ▸	E2		EE2		Genistein		BPA		Methylparaben		Butylparaben		Combination 1		Combination 2	
Varied ▾	**E**	**P**	**E**	**P**	**E**	**P**	**E**	**P**	**E**	**P**	**E**	**P**	**E**	**P**	**E**	**P**
E2			*	2.5 x10^{-11}	2.1 x10^{-11}	2.9 x10^{-11}	2.9 x10^{-11}	2.7 x10^{-11}	*	3.3 x10^{-11}	*	2.4 x10^{-11}	2.9 x10^{-11}	2.8 x10^{-11}	5.5 x10^{-11}	1.9 x10^{-11}
EE2	5.2 x10^{-13}	7.2 x10^{-12}			3.0 x10^{-12}	6.1 x10^{-12}	3.5 x10^{-12}	6.3 x10^{-12}	6.5 x10^{-12}	7.4 x10^{-12}	2.8 x10^{-12}	7.2 x10^{-12}	7.0 x10^{-12}	8.6 x10^{-12}	4.2 x10^{-12}	6.5 x10^{-12}
Genistein	5.7 x10^{-8}	8.0 x10^{-8}	3.2 x10^{-7}	8.3 x10^{-8}			9.3 x10^{-8}	8.8 x10^{-8}	3.9 x10^{-7}	1.1 x10^{-7}	1.2 x10^{--7}	8.3 x10^{-8}	1.9 x10^{-7}	1.1 x10^{-7}	2.5 x10^{-7}	7.0 x10^{-8}
BPA	2.2 x10^{-7}	7.6 x10^{-7}	3.5 x10^{-6}	2.8 x10^{-7}	3.0 x10^{-7}	6.7 x 10^{-7}			5.7 x10^{-7}	5.7 x10^{-7}	1.5 x10^{-7}	8.4 x10^{-7}	3.3 x10^{-7}	7.4 x10^{-7}	1.3 x10^{-6}	6.1 x10^{-7}
Methylparaben	4.6 x10^{-7}	3.7 x10^{-7}	2.3 x10^{-8}	4.0 x10^{-7}	3.3 x10^{-7}	3.9 x10^{-7}	1.3 x10^{-7}	3.7 x10^{-7}			5.1 x10^{--8}	4.0 x10^{-7}	1.3 x10^{-6}	5.4 x10^{-7}	3.4 x10^{-6}	2.7 x10^{-7}
Butylparaben	1.2 x10^{-7}	6.8 x10^{-7}	2.3 x10^{-5}	7.2 x10^{-7}	1.3 x10^{-7}	8.7 x10^{-7}	*	7.9 x10^{-7}	6.3 x10^{-6}	6.6 x10^{-7}			5.8 x10^{-8}	6.7 x10^{-7}	8.8 x10^{-7}	8.1 x10^{-7}

Table 5.6: Comparison of experimental (E) and concentration CA predicted (P) ERβ CALUX® EC_{50} values for the xenoestrogen combinations. * indicates a response was not converged using non-linear regression.

Fixed ▸	E2		EE2		Genistein		BPA		Methylparaben		Butylparaben		Combination 1		Combination 2	
Varied ▾	**E**	**P**	**E**	**P**	**E**	**P**	**E**	**P**	**E**	**P**	**E**	**P**	**E**	**P**	**E**	**P**
E2			2.2 x10^{-9}	1.2 x10^{-10}	5.0 x10^{-10}	4.1 x10^{-10}	5.4 x10^{-10}	5.7 x10^{-11}	2.7 x10^{-10}	2.1 x10^{-10}	8.1 x10^{-10}	2.0 x10^{-10}	3.5 x10^{-9}	3.3 10^{-11}	1.6 x10^{-9}	6.9 x10^{-10}
EE2	1.3 x10^{-9}	6.4 x10^{-10}			6.2 x10^{-10}	9.4 x10^{-10}	2.7 x10^{-9}	1.4 x10^{-10}	1.4 x10^{-9}	6.3 x10^{-10}	4.0 x10^{-9}	3.0 x10^{-9}	1.6 x10^{-11}	3.3 x10^{-10}	2.9 x10^{-9}	4.1 x10^{-9}
Genistein	*	1.2 x10^{-7}	2.1 x10^{-9}	3.1 x10^{-9}			6.6 x10^{-10}	8.3 x10^{-8}	3.5 x10^{-9}	1.1 x10^{-7}	1.3 x10^{-8}	1.4 x10^{-6}	1.8 x10^{-9}	9.6 x10^{-8}	1.8 x10^{-8}	9.6 x10^{-7}
BPA	8.3 x10^{-7}	9.9 x10^{-7}	5.5 x10^{-6}	7.6 x10^{-7}	1.7 x10^{-7}	9.5 x10^{-7}			1.1 x10^{-6}	9.9 x10^{-7}	3.5 x10^{-6}	5.1 x10^{-6}	2.0 x10^{-7}	8.5 x10^{-7}	2.0 x10^{-7}	*
Methylparaben	2.2 x10^{-5}	1.3 x10^{-5}	9.4 x10^{-5}	2.2 x10^{-5}	2.7 x10^{-5}	4.4 x10^{-6}	2.2 x10^{-5}	2.7 x10^{-6}			6.4 x10^{-5}	6.6 x10^{-5}	1.0 x10^{-5}	6.6 x10^{-6}	1.9 x10^{-6}	9.1 x10^{-6}
Butylparaben	4.4 x10^{-6}	9.1 x10^{-7}	2.3 x10^{-6}	5.3 x10^{-7}	1.7 x10^{-6}	1.0 x10^{-7}	6.9 x10^{-7}	6.7 x10^{-7}	2.3 x10^{-7}	1.0 x10^{-6}			3.4 x10^{-6}	5.0 x10^{-9}	3.7 x10^{-7}	3.8 x10^{-6}

Table 5.7: Calculated maximal luciferase response change (%) for xenoestrogen combinations compared to the varied component of the mixture in the ERα CALUX® assay. * indicates a response was not converged using non-linear regression (yellow). Green shading indicates a decreased maximal response and blue shading indicates an increased maximal response.

ERα Activation of xenoestrogen combinations (%)								
	Fixed ▼							
Varied ▼	E2	EE2	Genistein	BPA	Methylparaben	Butylparaben	Combination 1	Combination 2
E2		*	18.5	20.52	*	82.3	62.1	86.5
EE2	31.8		20.8	1.00	50.2	63.6	22.9	69.3
Genistein	64.7	6.9		80.65	37.0	22.7	63.0	1.3
BPA	22.3	25.7	12.6		52.0	62.2	65.5	63.5
Methylparaben	17.4	74.1	10.9	38.72		72.9	66.7	81.7
Butylparaben	59.6	0.9	27.9	44.19	13.9		58.3	66.2

Table 5.8: Calculated maximal luciferase response change (%) for xenoestrogen combinations compared to the varied component of the mixture in the ERβ CALUX® assay. Green shading indicates a decreased maximal response and blue shading indicates an increased maximal response.

ERβ activation of xenoestrogen combinations (%)								
	Fixed ▼							
Varied ▼	E2	EE2	Genistein	BPA	Methylparaben	Butylparaben	Combination 1	Combination 2
E2		47.3	7.1	63.7	51.9	10.0	28.3	4.8
EE2	5.8		40.1	50.2	47.2	27.6	49.6	12.0
Genistein	15.2	30.5		33.4	11.6	50.6	52.1	30.5
BPA	10.2	44.8	2.1		49.7	17.2	45.0	37.0
Methylparaben	19.9	38.4	10.9	55.2		41.9	41.0	24.6
Butylparaben	0.7	25.3	26.5	48.9	34.8		6.7	2.4

5.3.2.1. E2

Testing in the ERα CALUX® assay (Fig. 5.2 and Appendix 1) revealed the E2 combination with BPA to induce a supramaximal luciferase response of 122.4% compared to E2; however, combinations with genistein (79.3%) and butylparaben (17.2%) revealed a decrease in the supramaximal response when compared to the effect elicited by the individual mixture components. Data for the EE2 and methylparaben combinations could not be converged using non-linear regression in the graphpad prism software (data not shown). Interestingly, the remaining data sets did not have a good correlation between the experimentally determined luciferase responses and the corresponding predicted CA responses in the ERα CALUX® assay. In comparison, in the ERβ CALUX® assay (Fig. 5.2 and Appendix 2), combinations with EE2 (224.0%), BPA (228.9%) and methylparaben (187.0%) exhibited an increased supramaximal luciferase responses and poor correlations with the predicted CA responses. Both genistein (97.6%) and butylparaben (81.6%) combinations showed a decrease in the maximal luciferase response but had a good correlation with the corresponding predicted CA response.

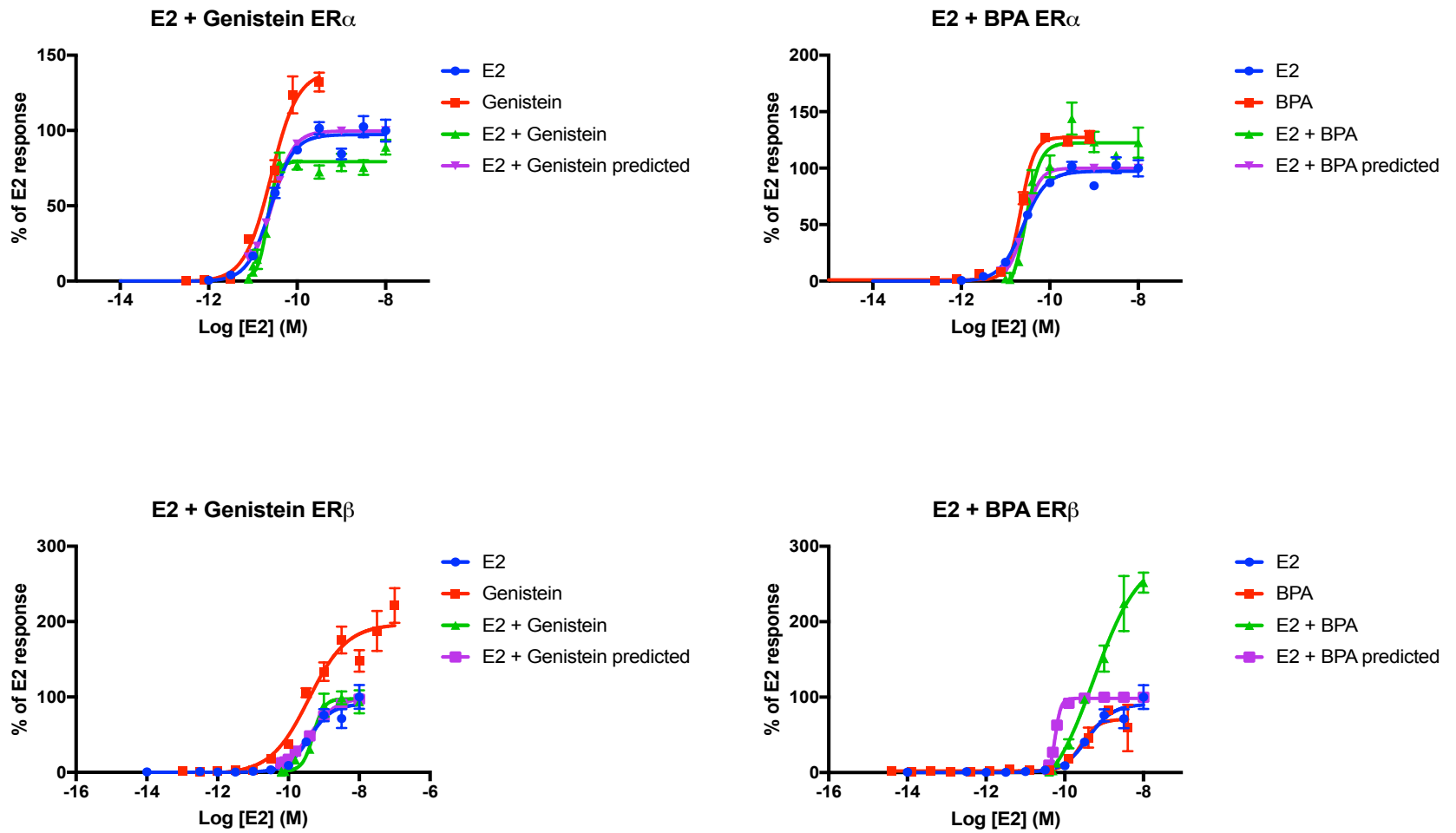


Figure 5.2: Example data for E2 (varied) in combination with genistein and BPA in both ERα and ERβ CALUX® assay. Results are presented using non-linear regression analysis with errors expressed as SEM.

5.3.2.2. EE2

Testing of EE2 in the two component combination studies showed a wide variety of responses compared with individual xenoestrogen responses. In the ERα CALUX® assay (Fig. 5.3 and Appendix 1) all combinations had a maximal luciferase response equal to or lower than the maximal luciferase response elicited by the individual components of the combinations. The only combination to have a confirmed good correlation with the predicted CA response was the BPA combination. All other responses had a poor correlation with the predicted CA responses. Interestingly, in the ERβ CALUX® assay (Fig. 5.3 and Appendix 2) combinations with BPA (224.0%) and methylparaben (211.2%) exhibited a supramaximal luciferase response and a poor correlation with the predicted CA responses. In comparison, E2, butylparaben and genistein combinations revealed a good correlation with the predicted CA response but genistein and butylparaben combinations showed a significant reduction of the supramaximal luciferase response compared to the individual xenoestrogen responses.

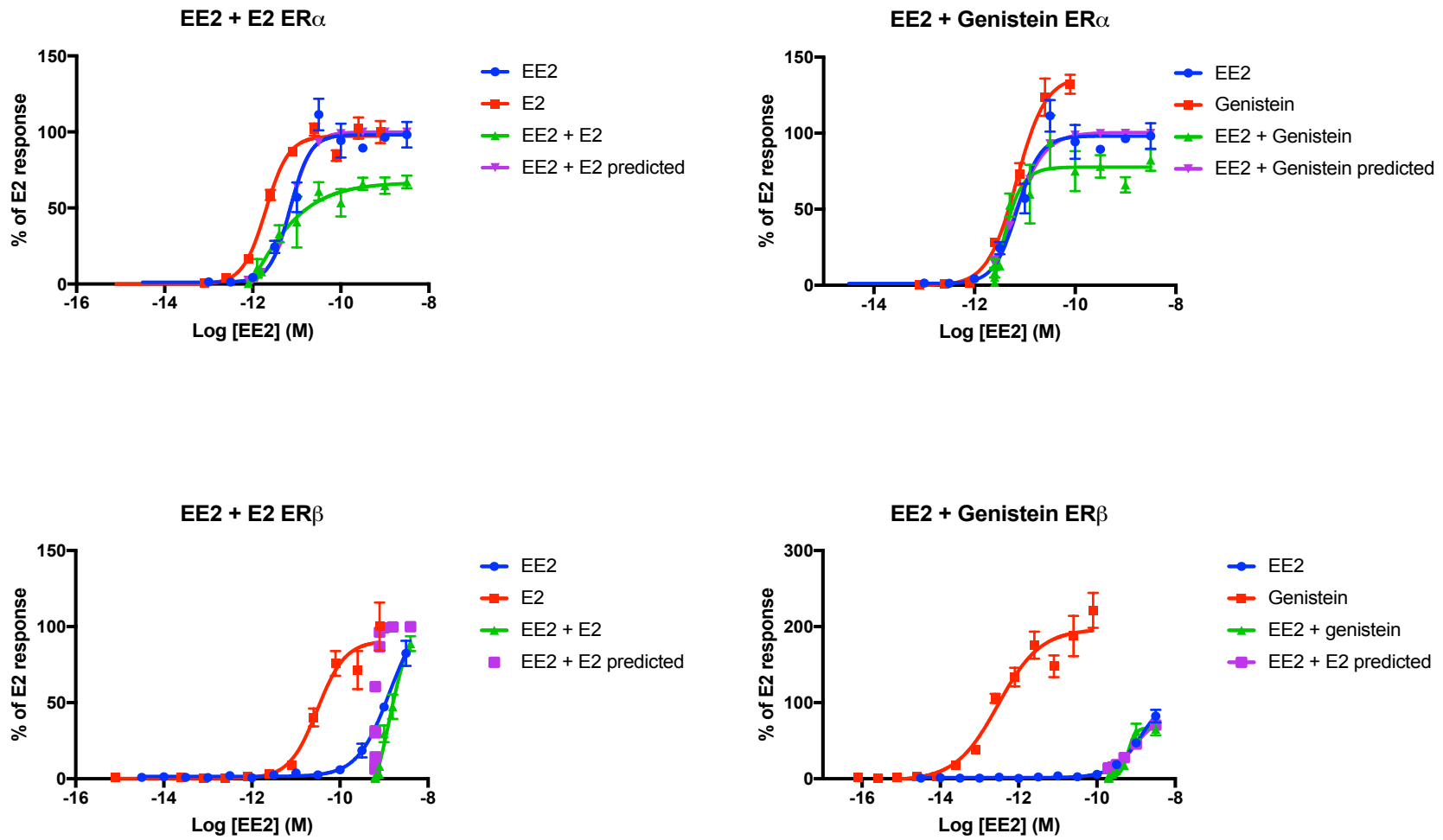


Figure 5.3: Example data for EE2 (varied) in combination with genistein and E2 in both ERα and ERβ CALUX® assay. Results are presented using non-linear regression analysis with errors expressed as SEM.

5.3.2.3. Genistein

Concentration response curves for genistein combinations also revealed a wide variety of responses in both ERα and ERβ CALUX® assays (Fig. 5.4 and Appendix 1). When tested in the ERα CALUX® assay, genistein (varied) combinations with E2, EE2 and methylparaben exhibited a supramaximal response compared to the individual responses of the mixture components; interestingly, these responses showed a good correlation with the predicted CA responses. In comparison, when the ERα CALUX® cells were exposed to genistein in combination with BPA the maximal response exceeded 200.0%, which is very much higher than either of the individual maximal luciferase responses and the predicted CA response. Exposure to genistein in combination with butylparaben (57.8%) resulted in a decrease in the maximal luciferase response compared with the individual xenoestrogen maximal responses. Also, a poor correlation between the experimental and predicted CA response was observed for the genistein + butylparaben combination. ERβ CALUX® cells responded differently to the genistein combinations, with supramaximal luciferase responses being observed in both methylparaben (222.9%; Appendix 2) and BPA (280.8%; Fig. 5.4) combinations. Interestingly, when ERβ CALUX® cells were exposed to combinations of genistein with E2, EE2 and butylparaben, a decrease in luciferase response of 221.3 %, 137.0 % and 93.3 % (Fig. 5.4 and Appendix 2), respectively, was observed. All ERβ genistein combinations had poor agreement with the corresponding predicted CA responses.

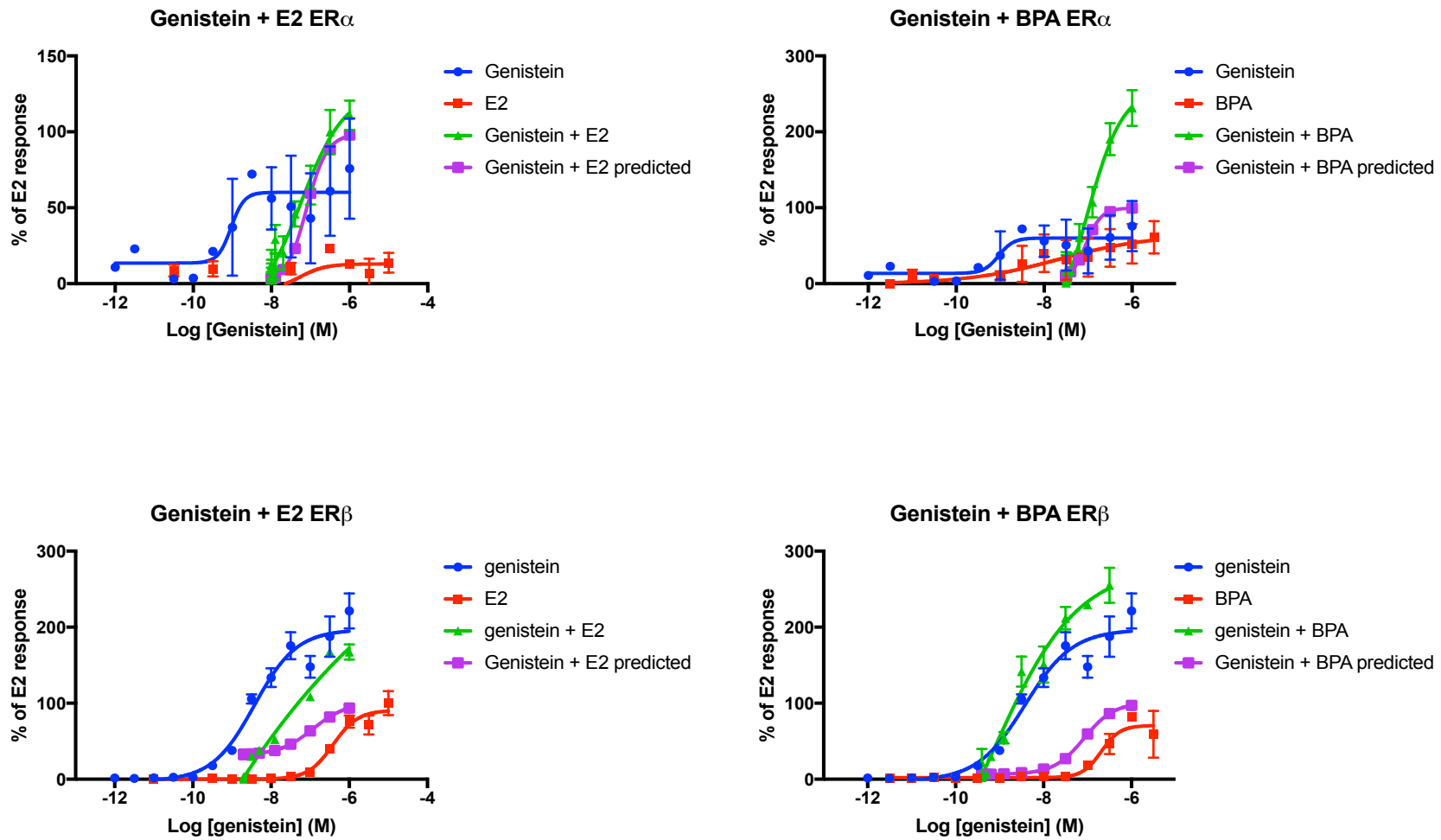


Figure 5.4: Example data for genistein (varied) in combination with E2 and BPA in both ERα and ERβ CALUX® assay. Results are presented using non-linear regression analysis with errors expressed as SEM.

5.3.2.4. BPA

Comparisons of the BPA containing combinations in both ERα and ERβ CALUX® (Fig. 5.5 and Appendices 1 and 2) assays revealed similar changes to the supramaximal luciferase responses observed in previous combinations. In the ERα CALUX® assay responses of BPA in combination with E2, EE2, methylparaben and butylparaben all elicited a decrease in the maximal luciferase response equal to or lower than the maximal E2 response (e.g. 100.0%). However, when ERα CALUX® cells were exposed to BPA in combination with genistein, a supramaximal luciferase response was observed. Furthermore, poor correlations were observed between the predicted CA responses and the corresponding combination in the ERα CALUX® cells. Studies in the ERβ CALUX® cells showed increased supramaximal responses with BPA (varied) in combination with EE2 (316.5%) and methylparaben (187.7%), while responses below 100% were observed for the remaining E2, genistein and butylparaben combinations; however, for both the genistein and butylparaben containing combinations the maximal luciferase response was significantly decreased when compared with the individual maximal responses of the mixture.

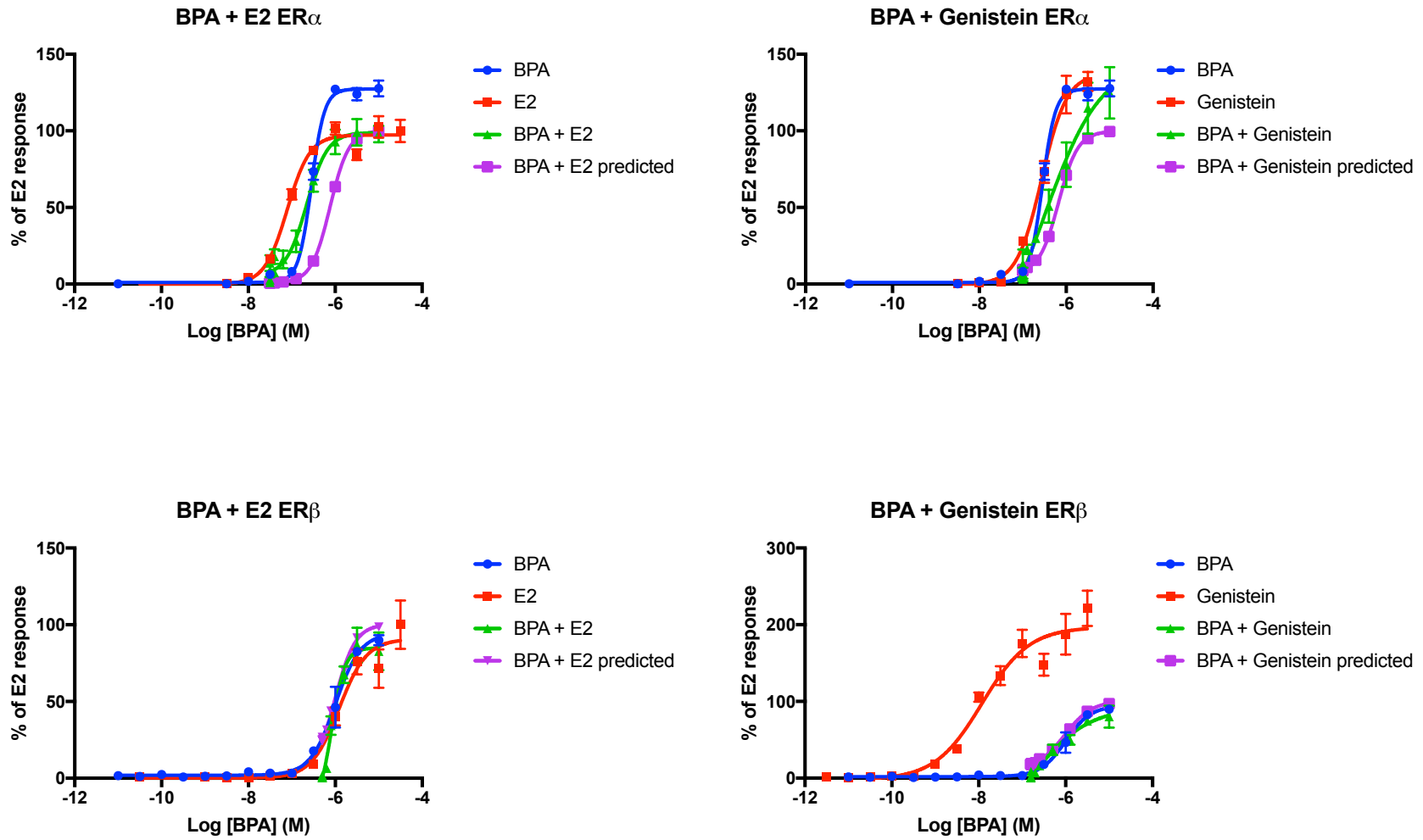


Figure 5.5: Example data for BPA (varied) in combination with E2 and genistein in both ERα and ERβ CALUX® assay. Results are presented using non-linear regression analysis with errors expressed as SEM.

5.3.2.5. Methylparaben

A decrease in the maximal luciferase responses to methylparaben-containing combinations with E2, EE2, genistein and butylparaben in the ERα CALUX® assay (Fig. 5.6 and Appendix 1) was observed in comparison to the maximal luciferase responses elicited by the corresponding individual xenoestrogens components of the mixture. A supramaximal response was observed from the methylparaben/BPA combination, with a maximal luciferase of 155.9% (Appendix 1). Poor agreement with predicted CA responses was observed for all ERα methylparaben combinations (Fig 5.6 and Appendix 1). Interestingly, the methylparaben/BPA combination exhibited a supramaximal luciferase response in the ERβ CALUX® assay (200.9%; Appendix 2), while the genistein combination exhibited a response of 100.9% (Fig. 5.6). On the other hand, the E2 combination's maximal luciferase response did not reach that of the maximal luciferase response of E2 alone or the predicted CA response. The methylparaben/butylparaben combination significantly decreased the maximal luciferase response observed in the ERβ CALUX® cells compared to butylparaben (176.0%), methylparaben (89.7%) and the predicted CA response (98.7%) (Fig. 5.6 and Appendix 2).

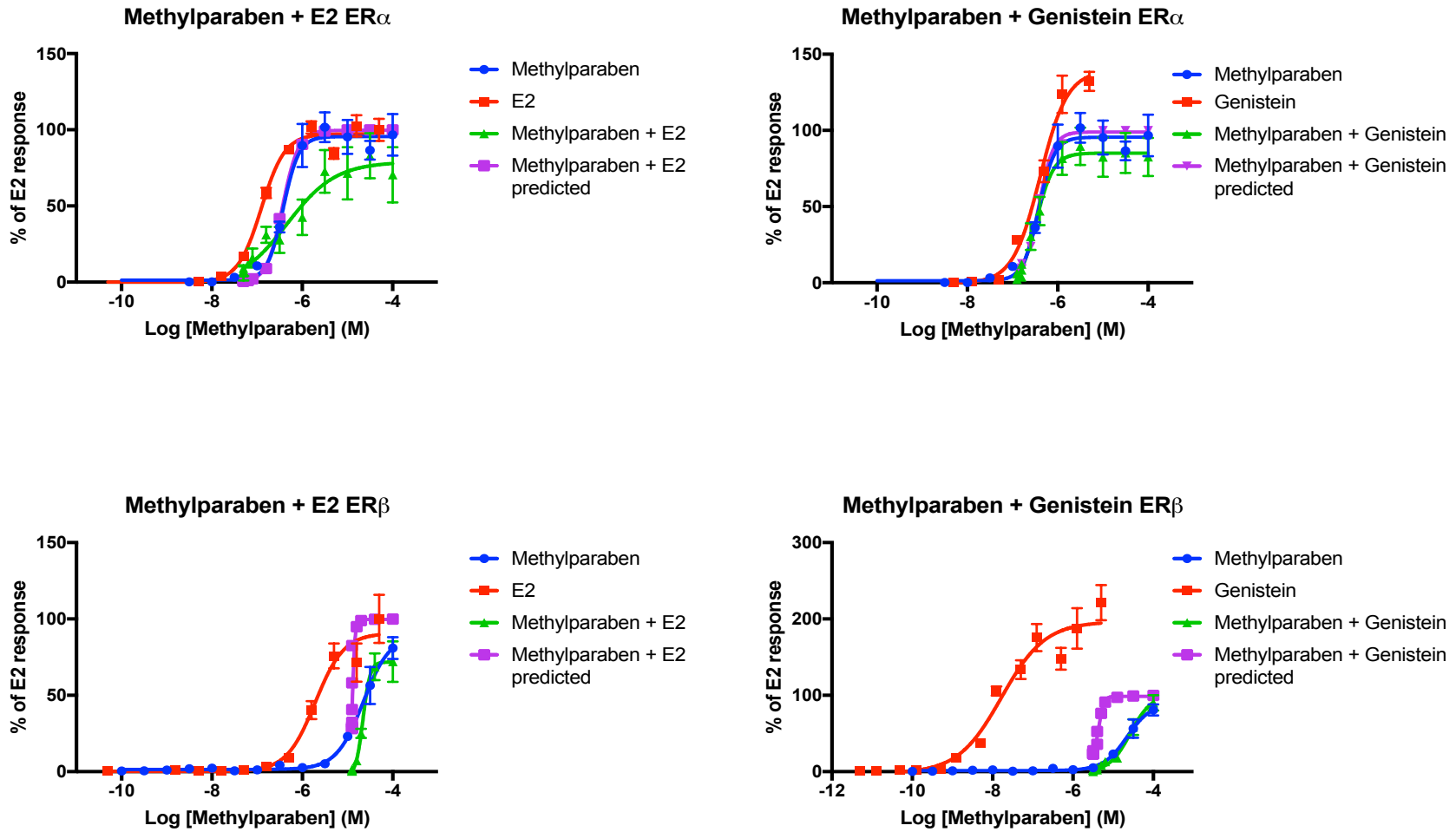


Figure 5.6: Example data for methylparaben (varied) in combination with E2 and genistein in both ERα and ERβ CALUX® assay. Results are presented using non-linear regression analysis with errors expressed as SEM.

5.3.2.6. Butylparaben

Supramaximal responses and poor agreement with the corresponding predicted CA responses were observed in butylparaben combinations with EE2 (327.1%), genistein (133.7%), BPA (354.2%) and methylparaben (215.1%) in the ERα CALUX® assay (Fig. 5.7 and Appendix 1). However, the butylparaben/E2 combination elicited a decreased maximal luciferase response compared to E2 (100%), butylparaben (185.3%) and the predicted CA response (98.9%). In comparison, in ERβ CALUX® assay (Fig. 5.7 and Appendix 2), supramaximal luciferase responses were observed in butylparaben combinations with E2 (177.3%), EE2 (235.5%), BPA (317.2%) and methylparaben (267.1%). However, when exposed to the butylparaben/genistein combination, the maximal luciferase response decreased compared to the maximal luciferase response of both butylparaben (176.0%) and E2 (90.7%). Poor correlations were also observed between all combinations tested in the ERβ CALUX® assay and the corresponding predicted CA responses.

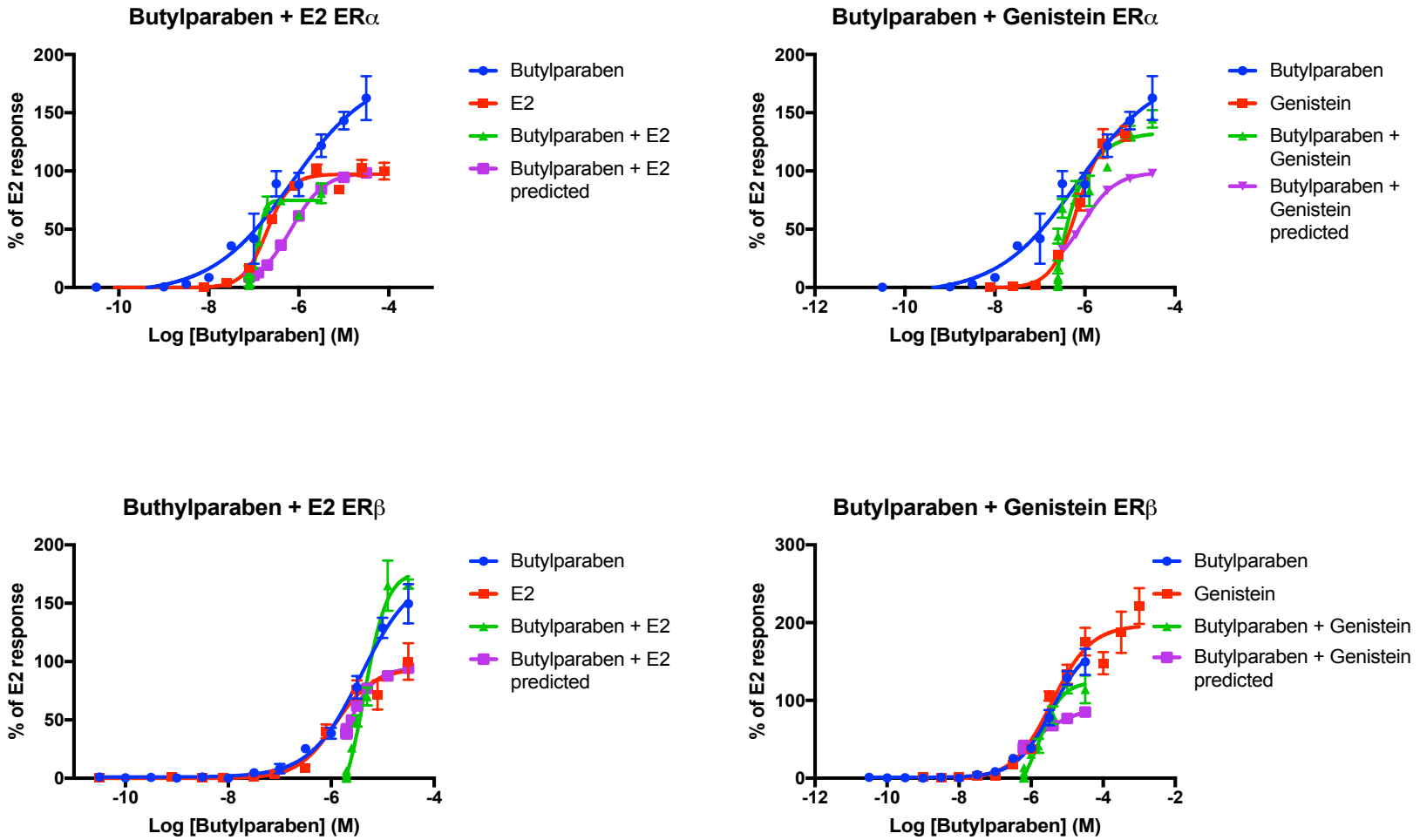


Figure 5.7: Example data for butylparaben (varied) in combination with E2 and genistein in both ERα and ERβ CALUX® assay. Results are presented using non-linear regression analysis with errors expressed as SEM.

5.3.3. Dose-response Analysis of Three Component Xenoestrogen Combinations

5.3.3.1. Combination 1

Comparisons between the responses of the ERα CALUX® assay (Fig. 5.8 and Appendix 3) revealed a decreased maximal luciferase response for combinations with E2 (36.9%), EE2 (52.7%), BPA (43.9%) and methylparaben (31.8%) to below 50.0% of the maximal luciferase response exhibited by E2. These responses all had a poor correlation with the corresponding predicted CA response. However, genistein and butylparaben combinations had good agreement with the corresponding predicted CA response and both exhibited a maximal luciferase response similar to E2 (e.g. 100.0%). Interestingly in the butylparaben combination, the supramaximal response elicited by individual xenoestrogens in the mixture disappeared when they were tested in combination. In comparison, in the ERβ CALUX® assay (Fig. 5.8 and Appendix), combinations with EE2 (68.8%), BPA (52.0%) and methylparaben (53.1%) all exhibited a slight decrease in the maximal response compared to the response for E2; therefore, poor agreement was observed between the predicted CA responses and the corresponding combination responses. In addition, both E2 and genistein combinations had a similar response to the corresponding predicted CA responses, thus, a similar maximal luciferase response was observed between both combinations and the individual E2 response. Interestingly, the butylparaben combination exhibited a supramaximal response and thus also had poor agreement with the predicted CA response.

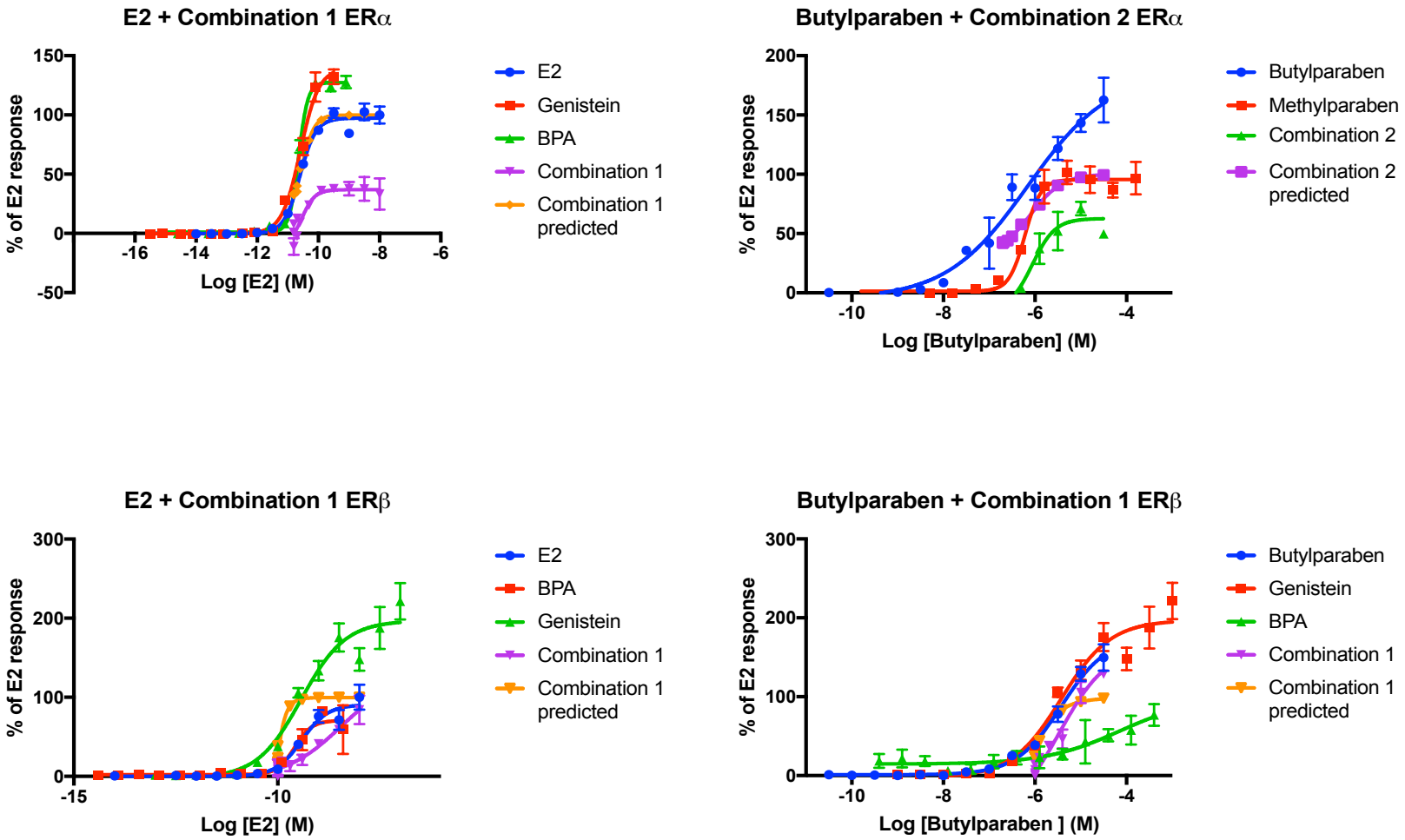


Figure 5.8: Example data for E2 and butylparaben (varied) in combination with genistein and BPA (fixed) in both ERα and ERβ CALUX® assay. Results are presented using non-linear regression analysis with errors expressed as SEM.

5.3.3.2. Combination 2

Combinations with E2 (13.1%), EE2 (30.1%), genistein (44.1%), BPA (46.5%) and methylparaben (17.5%) all exhibited a lower luciferase maximal response compared with the response to E2 in the ERα CALUX® assay (Fig. 5.9 and Appendix 3). Therefore, poor agreement between the responses and the corresponding predicted CA responses was observed. However, the butylparaben combination exhibited a similar response to its corresponding predicted CA response. Interestingly, the supramaximal response observed for butylparaben alone disappeared when tested in combination with the other mixture components (Fig. 5.9). In the ERβ CALUX® assay (Fig. 5.9 and Appendix 3), good correlations were observed between maximal luciferase responses for the E2, EE2 and methylparaben combinations and the predicted CA responses. However, supramaximal responses were observed for genistein (137.0 %), BPA (120.5 %) and butylparaben (137.2 %) combinations and obviously poor correlations between predicted CA responses and experimental responses were observed.

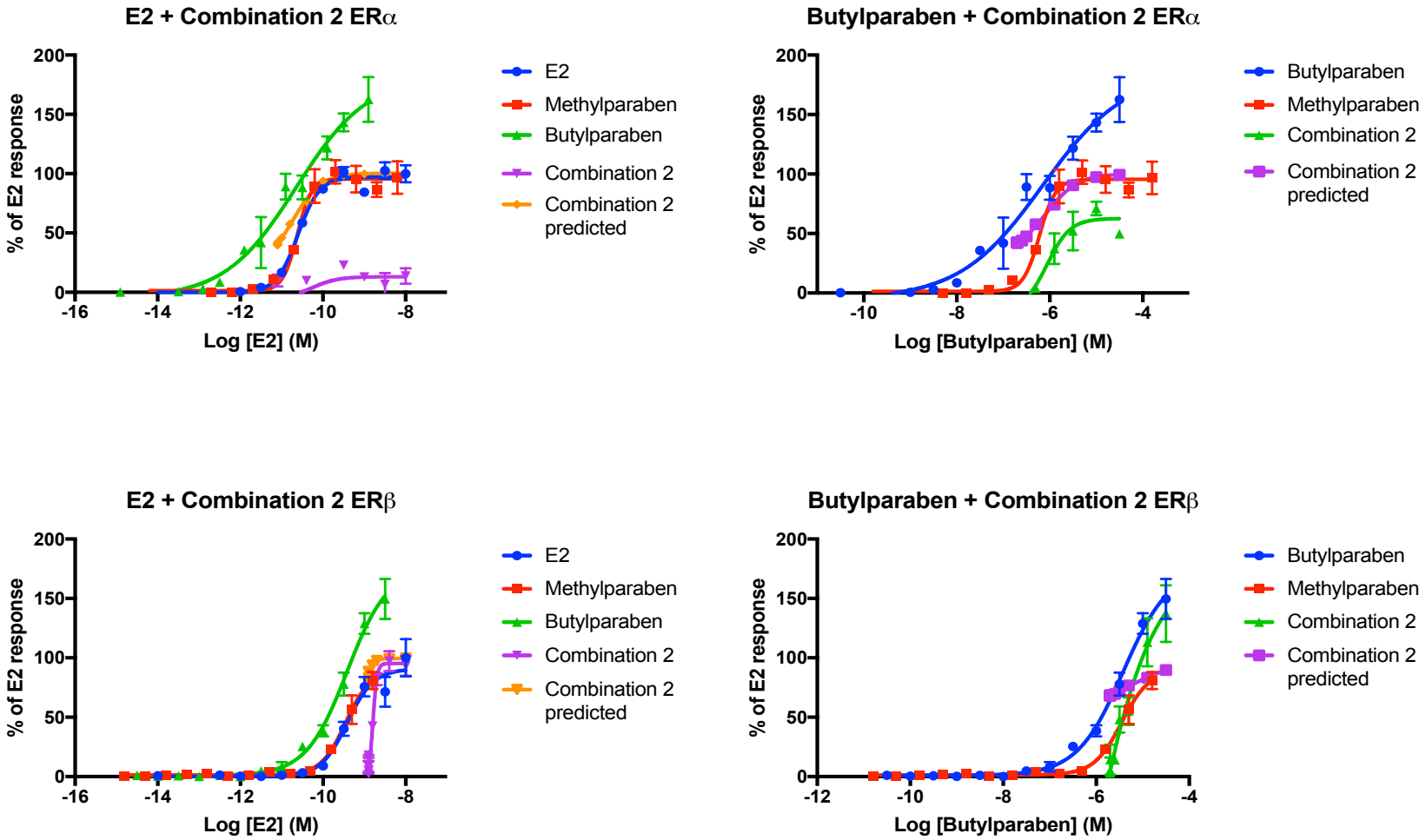


Figure 5.9: Example data for E2 and butylparaben (varied) in combination with genistein and BPA (fixed) in both ERα and ERβ CALUX® assay. Results are presented using non-linear regression analysis with errors expressed as SEM.

5.3.4. Cytotoxicity Testing

Cytotoxicity testing was carried out for individual xenoestrogens and three component studies. The same concentration combinations were used for cytotoxicity testing as in the ERα and ERβ assays (see Tables 5.2 and 5.3). A positive result for cytotoxicity is indicative by a decrease in luciferase response. Therefore, a cytotoxic response was observed for methylparaben alone and methylparaben and EE2 each with combination 2 (ERα). Interestingly, individual exposures and combinations with genistein and butylparaben both exhibited increased luciferase responses in the cytotox CALUX® assay. In addition, E2 with combination 1 (ERα) and combination 2 (ERβ), methylparaben with combination 2 (ERβ), and BPA with combination 2 (ERβ) (Table 5.3) all exhibited increase luciferase responses (Fig. 5.10). This suggests that cytotoxicity did not interfere with most of the results presented above for the ERα and ERβ CALUX® assays. It also suggests that the supramaximal effects observed in the ERα and ERβ CALUX® assays were not directly related to ER-mediated transcription but rather might be explained by an artefact of the assays (see Section 5.4.2.).

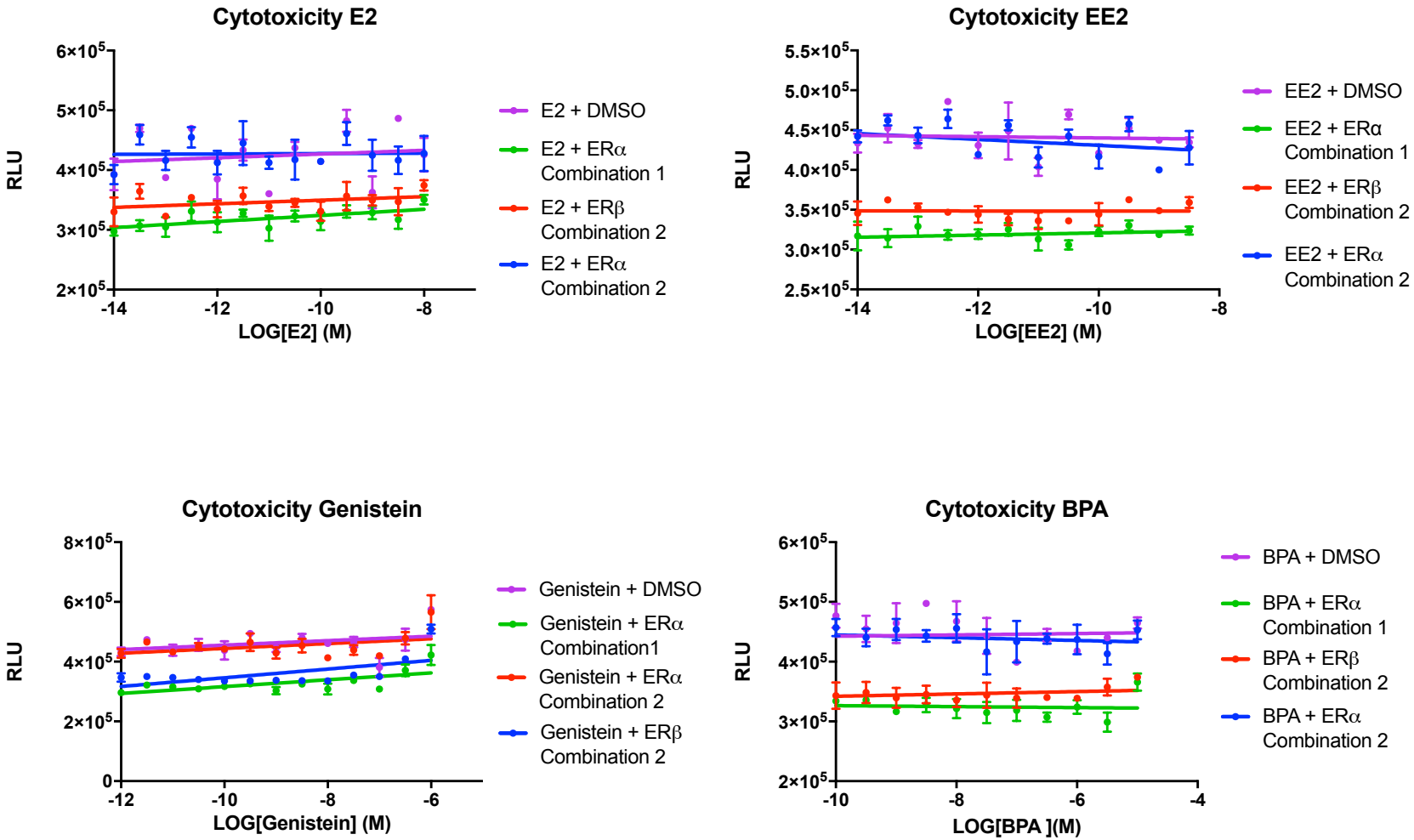


Figure 5.10: also see page 171

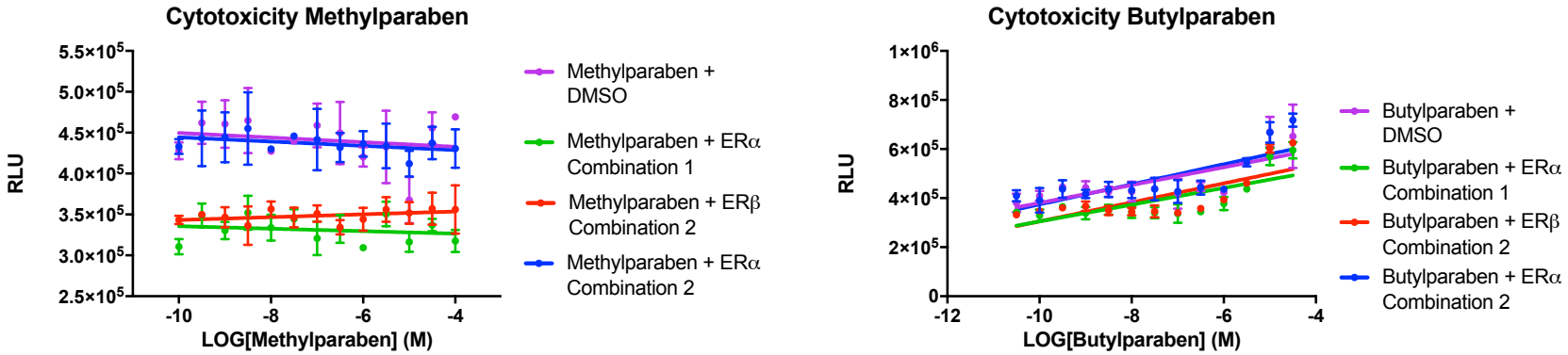


Figure 5.10: Results of individual and selected combinations of xenoestrogens in the cytotoxicity CALUX® assay. Results are presented using linear regression analysis with errors expressed as SEM.

5.4. Discussion

In this chapter, the estrogenic potencies of individual and combinations of xenoestrogens were examined in both the ERα and ERβ CALUX® assays. The results showed a wide range of responses, including supramaximal responses in both individual and combination experiments, with poor correlations between combination responses and their corresponding predicted CA responses.

5.4.1. Estrogenicities of Individual Xenoestrogens

As discussed above (Section 5.1) there has been a considerable effort over the last two decades to develop *in vitro* assays capable of screening and assessing the estrogenic activity of xenoestrogens. During this time a wide range of xenoestrogens has been tested to determine the individual estrogenic potencies, however, few studies incorporate ERβ in the test regimes, especially when assessing the estrogenic effects of xenoestrogens in a breast cancer context. Since the ERβ may act as a counterpart of the ERα, it is of great relevance for the risk assessment process to limit the analysis of mixture effects to ERα, especially since most ERα ligands also bind ERβ. Indeed, some xenoestrogens (e.g. phytoestrogens) have a higher binding affinity for ERβ compared to ERα, highlighting the importance of the intracellular ER receptor ratio in the breast. This is evident from the results presented in this chapter with the individual xenoestrogen responses in both ERα and ERβ exhibiting a wide range of potencies. Therefore, to give a more precise comparator, estrogen equivalents (EQs) were calculated for xenoestrogens (X) from the EC_{50} values as follows (Table 5.9) (Soto*, et al.*, 1997):

$$\text{EQ} = \frac{EC_{50}\ [E2]}{EC_{50}\ [X]}$$

The EQ values clearly show that EE2 is the most potent xenoestrogen studied, indeed it is 3.54-fold more potent than E2 in ERα. In addition, there are significant differences between ERα and ERβ responses– EE2 is 12.24-fold more potent in ERα compared to ERβ. On the other hand, genisteins ERα/ERβ EQ ratio is 0.055 showing that it binds preferentially to ERβ. It is clear from these and previously published results that individual xenoestrogens will have different estrogenic effects on cells,

and further, will have different effects according to the ER isoform to which they preferentially bind. The toxicological implications of exposure to xenoestrogen cocktails depends on whether or not their effects are additive or perhaps synergistic (see Section 1.4.5.).

Table 5.9: Calculated estrogen equivalents (EQ) for individual xenoestrogens tested in the ERα and ERβ CALUX® assay. The ERα/ERβ binding preference is also shown.

	ERα EQ	ERβ EQ	ERα/ERβ ratio
EE2	3.5	0.3	12.2
Genistein	0.006	0.1	0.06
BPA	0.00009	0.0004	0.2
Methylparaben	0.00007	0.000002	37.7
Butylparaben	0.00004	0.0001	0.4

5.4.2. Estrogenicities of Xenoestrogen Combinations in the ERα and ERβ CALUX® Assay

The combination studies presented in this chapter exhibit a wide range of responses in both the ERα and ERβ CALUX® assays. Individually, genistein, butylparaben and BPA (ERα only) exhibit supramaximal effects; however, supramaximal effects were not seen in combinations with E2 and EE2 (i.e. high LBC binding ligands). Interestingly, in the presence of a lower LBC binding affinity ligand (e.g. methylparaben) the supramaximal effect was amplified. In addition, some ERβ CALUX® assay combination experiments exhibited a supramaximal effect (e.g. BPA + methylparaben) where no supramaximal effect was observed for the individual xenoestrogen responses. Therefore, for interpretation of the CALUX® results, it is important to understand whether this effect is related to the real supramaximal response of gene expression that might also occur *in vivo* or whether it is an artefact of the assay. For example, it has been suggested that supramaximal effects are a result of a direct luciferase interaction, whereby some xenoestrogens (e.g. genistein) stabilise the degradation of the enzyme, resulting in a supramaximal response – this will be discussed in more detail later.

The presence of supramaximal effects (effects that exceed the maximal effect elicited by the natural ligand, E2) has been noted in *in vitro* assays for over 20 years, with the

first reported in 1994 (Makela*, et al.*, 1994). However, supramaximal effects are not consistently reported in the scientific literature, and sometimes reports appear to be contradictory (Montano*, et al.*, 2010). Many authors only mention the presence of a maximal effect, while others do not describe supramaximal effects in their results or mention them in the discussion, even when a supramaximal response is apparent. Other authors will acknowledge the presence of a supramaximal effect and normalise the results, so they cannot exceed 100% activation of the natural ligand E2 (Jonker*, et al.*, 2005, Murk*, et al.*, 1996, Vrabie*, et al.*, 2009). Several xenoestrogens, including genistein, have been reported to induce a supramaximal response in luciferase reporter gene assays. Many possible explanations have been put forward over the years, including an ER-mediated response, and post-transcriptional mechanisms such as a non-ER-mediated mechanism and stabilisation of luciferase leading to an increased half-life of the enzyme and thus a supramaximal response.

Sotoca *et al*., (2010) extensively investigated some of the possible mechanisms that might explain the supramaximal response. They found much higher concentrations of ER antagonists were required to block the supramaximal luciferase induction by genistein compared to E2. This suggests that, although higher concentrations of inhibitor are required to inhibit induction of luciferase, the supramaximal effect induced by genistein is ER-mediated. However, they also found that the supramaximal effect induced by genistein in reporter gene assays is not mirrored at the cell proliferation level (e.g. superproliferation). This illustrates that the phenomenon of the supramaximal effect is not reflected at the biologically relevant endpoint (e.g. *in vivo*) and therefore, it is likely an artefact of the assay.

This is further supported by Sotoca and colleague's studies which examined the correlation between luciferase activity and real luciferase mRNA induction, to determine whether the supramaximal effect was a true reflection of transcriptional activity. Interestingly, no correlation was found between luciferase activity and mRNA induction when exposed to genistein. This suggests that xenoestrogens that induce a supramaximal effect are doing so via an alternative mechanism.

Finally, the possible direct interaction between genistein and the luciferase enzyme was examined. It had been previously proposed that certain compounds can directly bind to, and stabilise, the luciferase enzyme, thereby increasing the half-life. Sotoca

and colleagues showed that in a cell-free biochemical assay, genistein and resveratrol (a xenoestrogen found in red grapes) stabilised the luciferase enzyme. Therefore, the increased bioluminescent signal induced during the cell-based assays is likely a result of luciferase stabilisation.

This stabilisation hypothesis likely explains the supramaximal effects observed in the results presented in this chapter for both the ERα and ERβ CALUX® assays. However, it does not explain the findings for all of the combinations that were tested. Some combinations did not induce a supramaximal luciferase response even though at least one xenoestrogen capable of inducing such effect was present. For example, when E2 (varied) was tested with genistein (fixed) the maximal luciferase response was 78.9% compared to the individual responses for E2 (100.0%) and genistein (132.2%). In theory, with genistein being the lower LBC binding affinity ligand in the combination, it would be expected that at higher concentrations E2 would outcompete genistein for the LBC, resulting in an increase in unbound genistein; therefore, more genistein would be freely available to stabilise the luciferase enzyme, inducing a supramaximal effect. However, the maximal luciferase response not only suggests that there is no genistein available to stabilise the luciferase enzyme, but also that the presence of genistein in the mixture decreases the ER-mediated transcription of the luciferase itself.

A possible explanation for this phenomenon is the potential of a two-site binding model, as proposed in the previous chapter (Chapter 4). The lack of the supramaximal response suggests that genistein is bound to a protein in the cell that was not previously available when genistein was tested individually. Therefore, if E2 could be inducing a conformational change at the LBC, making binding at the AF-2 site more favourable for genistein, not only would a supramaximal effect no longer be observed, but also a decrease in transcription of the luciferase enzyme via a possible disruption of coactivator protein recruitment at the ER (Fig. 5.11).

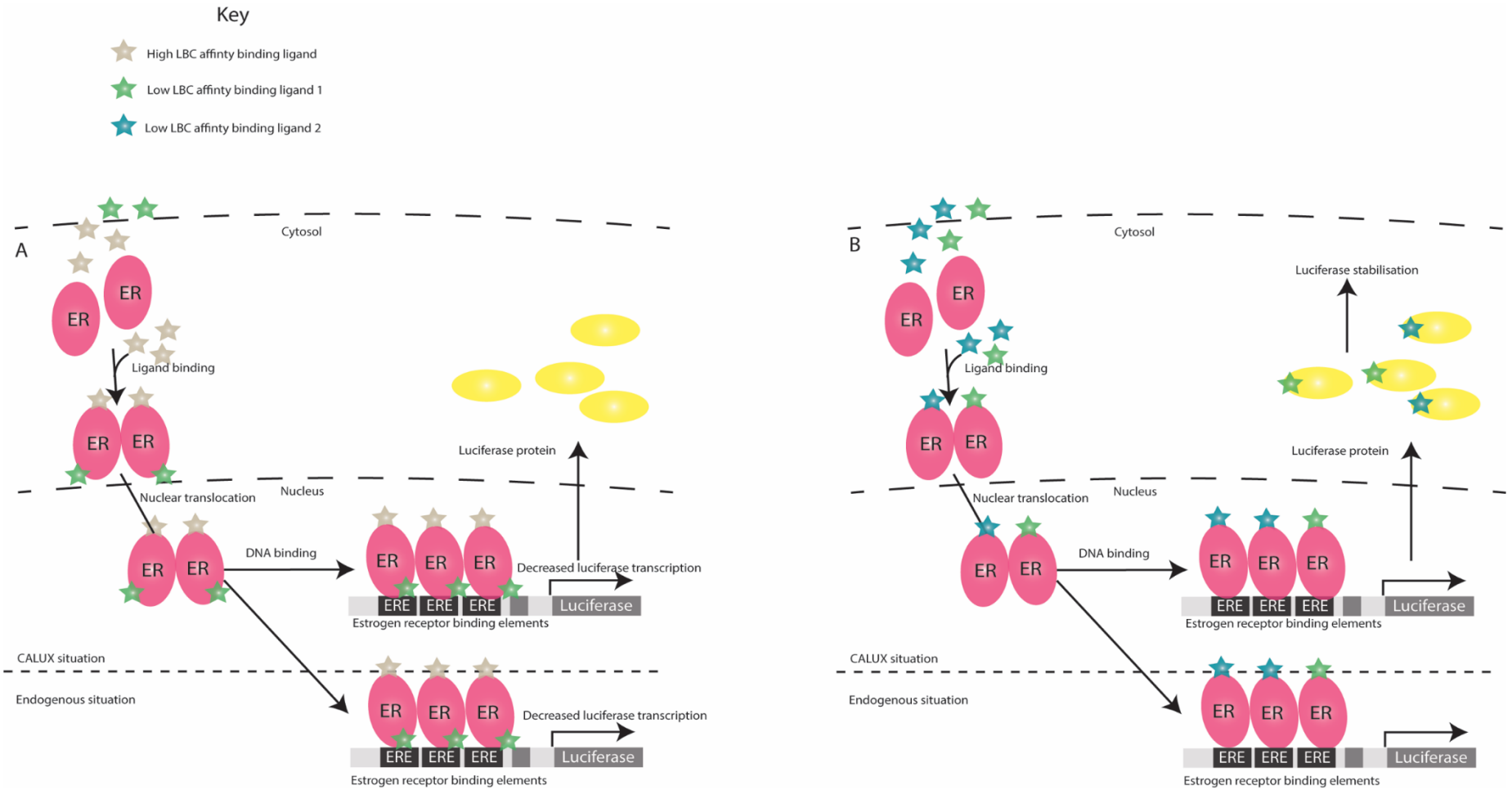


Figure 5.11: Proposed two-site model for xenoestrogens (, and) in the ERα and ERβ CALUX® assays. Two mechanisms are proposed: (A) shows the decreased luciferase response as a result of a high affinity and in combination with a low affinity LBC binding ligand and (B) shows an increased luciferase response (supramaximal) as a result of a xenoestrogen mixture with two low affinity LBC binding ligands. (Elements of this diagram were derived from BioDetection Systems Ltd. The concept of the entire diagram is the authors).

However, not all combinations exhibited this effect, and an increased supramaximal response was observed in many lower LBC binding affinity ligand combinations. For example, the combination with butylparaben (varied) and BPA (fixed) induced a supramaximal effect of 332.9% compared to the individual maximal luciferase responses for butylparaben (162.6%) and BPA (122.6%). The significantly increased supramaximal response is not surprising considering both butylparaben and BPA induce supramaximal effects individually; therefore, it would be expected that in combination there would be a higher supramaximal response due to the increased presence of unbound xenoestrogens.

Furthermore, genistein and butylparaben both exhibited increased supramaximal luciferase responses in the cytotox CALUX® assay, which further supports the hypothesis of luciferase enzyme stabilisation by these compounds. All genistein and butylparaben combinations exhibited the same increased luciferase response in the cytotoxicity assay in addition to methylparaben, BPA and E2 in combination with BPA butylparaben and methylparaben and E2 and EE2 in combination with BPA and genistein. Interestingly, for almost all of the combinations that exhibited an increased luciferase response in the cytotoxicity assay, a decrease in the maximal luciferase responses was observed in the ERα and ERβ CALUX® assays. This suggests that the luciferase enzyme stabilisation that was observed in the cytotoxicity assay is not occurring. Since the only difference between the cytotoxicity assay and the ERα and ERβ assays is the presence of the ER, it is likely that the decrease in the maximal luciferase response is ER-mediated. This also aligns with the possible two-site binding model proposed above, where only in the presence of an ER will a decrease in the supramaximal effect be observed.

It is clear that the changes in supramaximal response are mixture-dependent, in that only certain mixtures elicit supramaximal responses, while other mixtures have a submaximal (e.g. below 100.0%) response. A possible explanation for this mixture-dependent response is the availability of the AF-2 site. Indeed, when comparing the ERα surface topography of E2, BPA and butylparaben-bound receptor it is clear that there are differences in charge distribution depending on the bound ligand (Fig. 5.12); therefore, the subtle differences in ligand binding at the LBC appear to have a significant influence on AF-2 surface topography. Thus, if ligands are binding at the

AF-2 site, the binding at the LBC could influence these interactions and account for the differences observed in the mixture-dependent supramaximal responses.

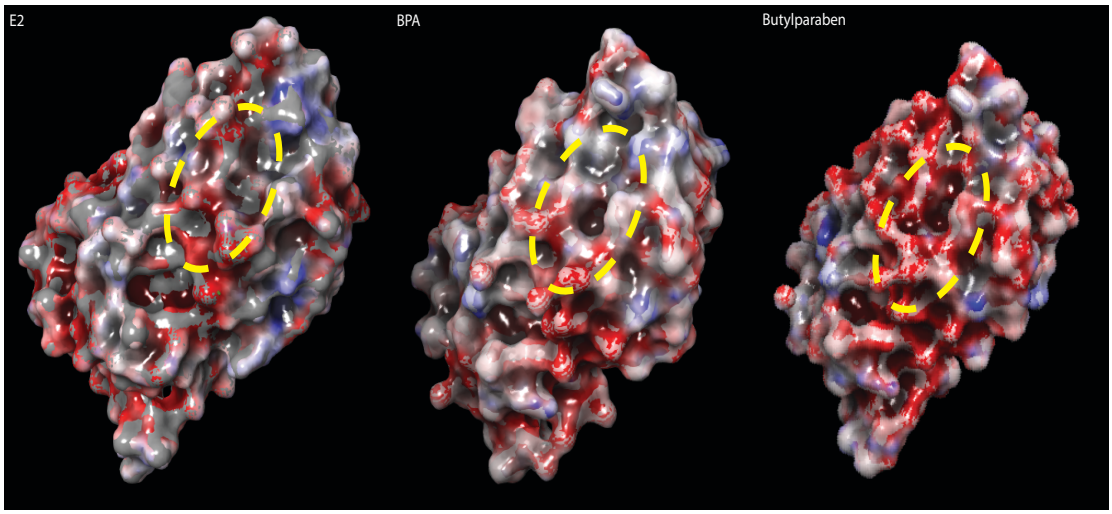


Figure 5.12: Illustration of the electrostatic charges on the surface of ERα complexed with E2 (left; from 5HYR), BPA (middle; from 3UU7) and butylparaben (right; from 4MG9). Blue represents negative and red represents positive electrostatic charges. Note a higher charge is indicated by a higher intensity of colour, while grey represents a neutral charge. Yellow dashed circles represent the approximate AF-2 binding site.

Indeed, E2 binding to the ER is known to induce a specific conformational change in the tertiary structure of the ER, which subsequently affects the alignment of the highly conserved amphipathic a-helix (H12) within AF-2 (Danielian*, et al.*, 1992). The correct agonist alignment of H12 exposes amino acid residues at the AF-2 site that interact with coregulatory proteins (Klinge*, et al.*, 1996). In addition, it has been shown that xenoestrogens can induce distinct conformational changes in the tertiary structure of the ERs following ligand binding at the LBC, likely a result of differences in steric and electrostatic properties of the various ligands (Paige*, et al.*, 1999, Routledge*, et al.*, 2000). Furthermore, it has been reported that different xenoestrogens recruit different coregulatory proteins at the ERs. For example, Routledge and colleagues found BPA to recruit fewer coregulatory peptides (e.g. peptides derived from coregulatory proteins) compared with E2 (Routledge*, et al.*, 2000). Interestingly, they also found ERβ had an enhanced ability to recruit coregulatory proteins compared with ERα in the presence of xenoestrogens, suggesting clear ER isoform differences. Indeed, this might explain the mixture-dependent supramaximal effects in the ERα and ERβ CALUX® assay studies

presented in this chapter. I suggest that differential ligand binding at the LBC explains the changes in supramaximal responses by recruitment of ligands to AF-2 as a secondary binding site on the ER ligand binding domain.

5.4.3. CA Correlations

In a natural exposure scenario, it is not possible to experimentally test every possible xenoestrogen mixture; therefore, mathematical models such as CA, are often used to predict the effects of mixtures. Researchers have concluded that CA most accurately predicts mixture effects, assuming that the mixture components act by a similar mode of action (e.g. the ER's LBC). However, there are inherent mathematical limitations to the CA model, most importantly the inability to predict mixture effects that elicit a maximal response greater than 100.0% (Scholze*, et al.*, 2014). This inability to deal with responses greater than 100.0% leads to predictive blind zones, and thus poor mixture predictability (Liu*, et al.*, 2015). This could explain the under-reporting and inconsistencies across the literature for supramaximal effects (Montano*, et al.*, 2010). However, it is clear from the results in this chapter that the supramaximal effects are an important aspect of a mixture's response. By not limiting the maximum luciferase response to 100.0%, it has led to an inability to predict mixture effects in this chapter. In general, poor correlations were observed between experimental and predicted EC_{50} values (Fig. 5.13), suggesting that the CA model may not be as robust in predicting xenoestrogen mixture effects as first thought. Indeed, the CA model assumes a similar mode of action for different components of a mixture; therefore, in my opinion, the responses presented in this chapter are a result of more than one mode of action (e.g. the two-site model) and therefore it was not surprising to find poor correlations between experimental and predicted EC_{50} values.

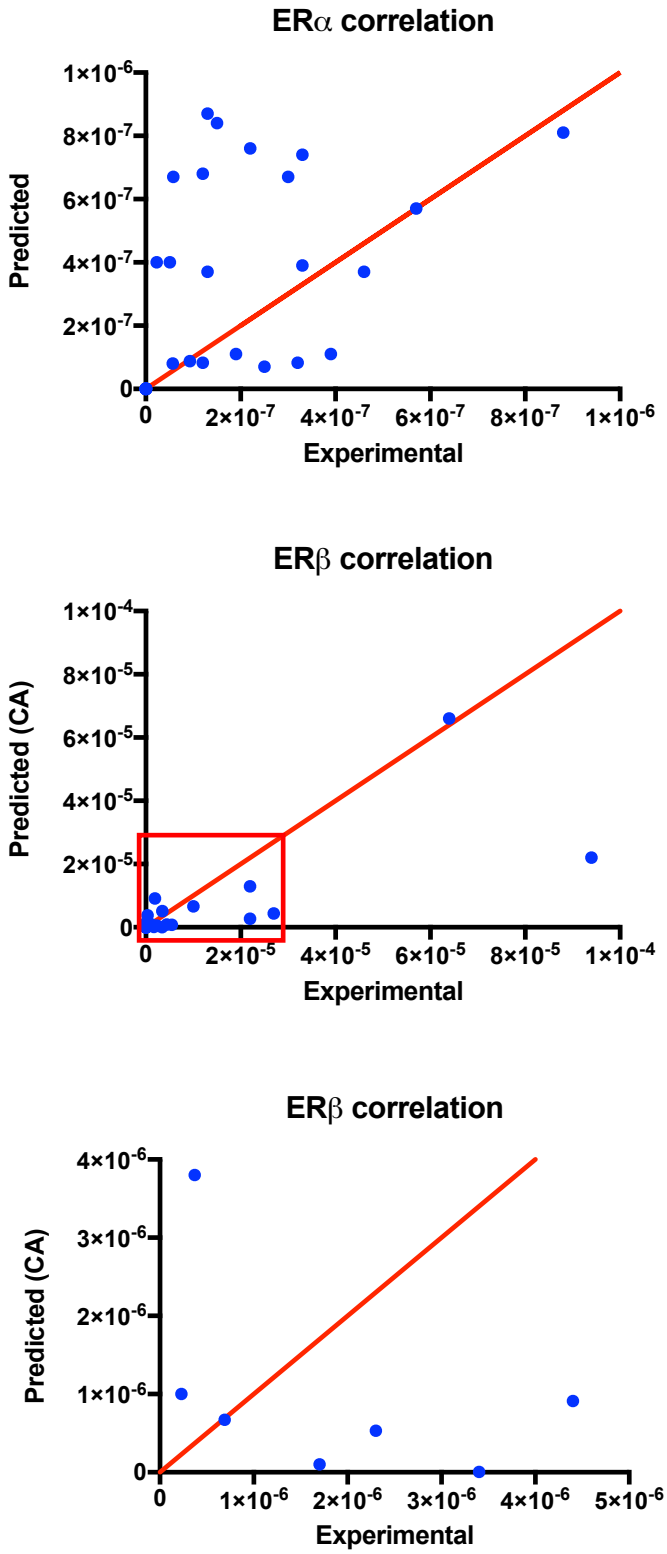


Figure 5.13: Correlation of experimental EC_{50} values and concentration addition (CA) predicted EC_{50} values in the ERα (top) and ERβ (middle) CALUX® assay. The second ERβ correlation (bottom) shows a zoomed in view of the responses in the red box from the above graph. The solid red line represents a 1:1 correlation.

The consequence of the unpredictability by CA and the uncertainty of the supramaximal phenomenon could result in over- or under-estimation of the estrogenic potency if the supramaximal effect is a molecular artefact of the cell model.

5.5. Concluding Remarks

From the results presented in this chapter, it is clear that simple additivity was not observed for all xenoestrogen combinations. The results from the mixture studies support the growing consensus (Evans*, et al.*, 2012) that additivity (CA) occurs when mixtures of xenoestrogens bind to the LBC. Genistein and butylparaben induced a supramaximal effect in the ERα and ERβ CALUX® assays; however, this phenomenon was not observed in all of the mixtures. Whilst the supramaximal effect is likely an artefact of the assay, the reduction in luciferase response appears to be ER-mediated. In my opinion this is likely a result of the two-site binding model previously proposed in Chapter 4, whereby some xenoestrogens could also bind to the AF-2 site on the ER, downregulating ER-mediated transcription and thus, E2-mediated cellular functions. Finally, the consideration of the ER-mediated reduction in luciferase response is necessary to accurately predict the outcomes of complex xenoestrogen exposure scenarios. In order to study this, investigations into LBC and AF-2 interactions will need to be performed to understand the interplay between these two binding sites. The Schrödinger computational docking platform was used to study these interactions in Chapter 6.

Chapter 6 *In silico* Predictions of Xenoestrogen Interactions with ERs

6.1. Introduction

A wide range of adverse outcomes in humans have been associated with exposure to xenoestrogens as presented in Chapter 1; an increased risk of developing breast cancer is one of the most significant health effects thought to be a result of exposure to environmental and dietary xenoestrogens. However, this hypothesis remains controversial with limited, often inconsistent, evidence reported on the effects of xenoestrogens, in particular phytoestrogens. As previously discussed, phytoestrogens may also have beneficial effects on human health which could reduce breast cancer risk. Indeed, it is clear from Chapters 4 and 5 that some xenoestrogens exert concentration-dependent agonist/antagonist effects, which appear to be ER-mediated; thus, understanding the mechanism is important for predicting the biological outcomes of xenoestrogen exposure cocktails. Previously I have speculated that the agonist/antagonist effects of some xenoestrogens are mediated by a two-site binding model, in which the agonist effects are mediated by LBC binding and the antagonist effects are mediated via AF-2 interactions. Therefore, the aim of this chapter is to investigate the interplay between xenoestrogens and the ER LBD binding sites.

6.1.1. Theoretical Background of the ER Binding Mechanism of Action

The binding of the ligand to the LBC represents a key priming step for the subsequent binding of ER, via the DBD, to gene promoters. Importantly, the LBD binds to E2 as a monomer with a twofold symmetry, wherein each monomer is largely comprised of a triple-layered antiparallel α-helical sandwich capped at one end by a small two-stranded anti-parallel β-sheet (Brzozowski*, et al.*, 1997). Within this α-helical sandwich, the central layer comprises H5, H6, H9 and H10 α-helices, flanked by one layer of H1, H3 and H4 α-helices on the left and one layer of H7, H8 and H11α-helices on the right as viewed from the direction of the sole β-sheet or the C-terminal α-helix H12 (Fig. 6.1). Subsequently, the ligand binds within the core of the globular

LBD with recognition of E2's A and D ring. This is achieved through intermolecular hydrogen bonding of E2's 3 OH with the carboxylate moiety of the glutamate residue from H3 and the guanidinium group of arginine residue from H5 as well as the E2's 17β-OH with imidazole sidechain of the histidine residue from H11. Additionally, van der Waals interactions are established as a result of sandwiching of the A/B-ring interface of E2 with amino acid residues alanine and leucine on one side and phenylalanine on the other side, as well as sandwiching of the E2's D ring with an isoleucine residue on one side and a leucine residue on the opposite side (Fig. 6.2) (Farooq, 2015).

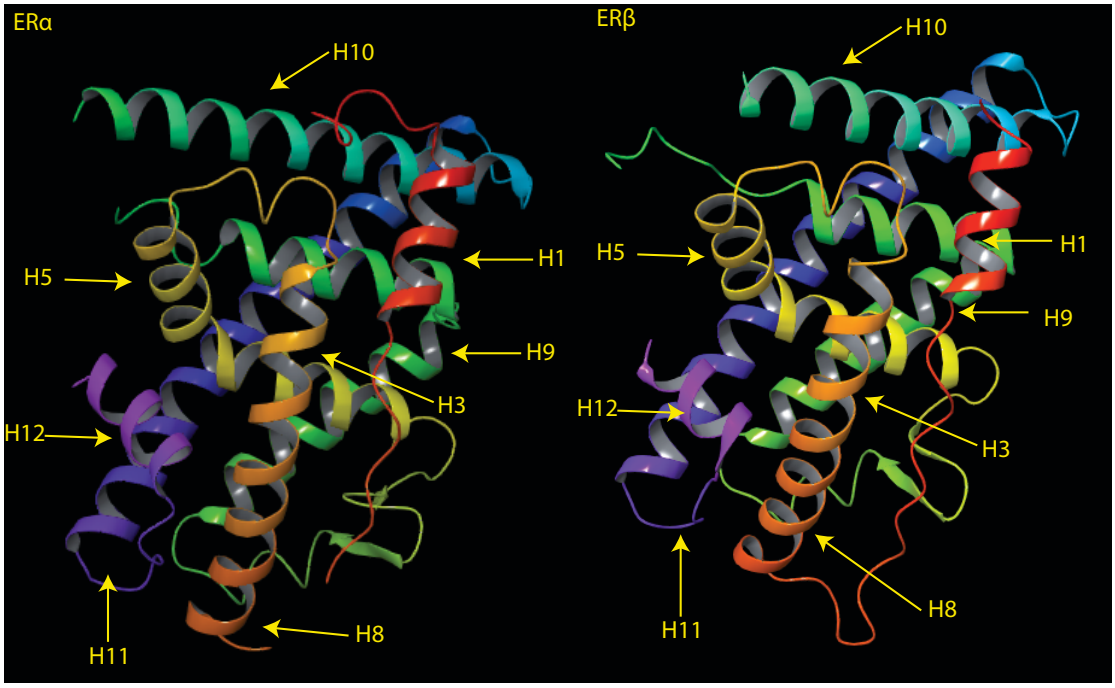


Figure 6.1: Crystal structures of ERα (PDB: 1ERE; left) and ERβ (PDB: 3OLS; right) with labelled α-helices.

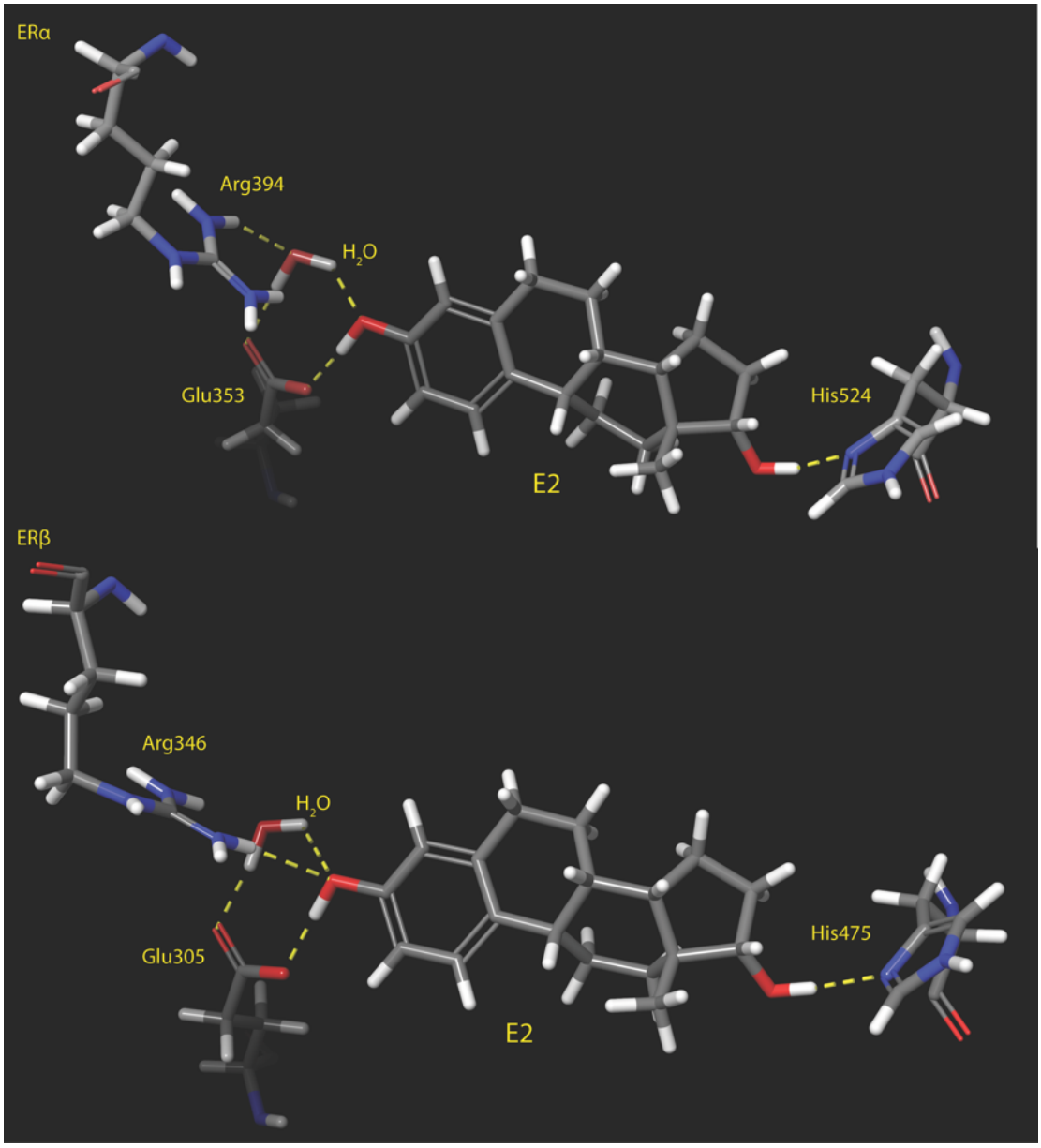


Figure 6.2: Key amino acid residue interactions at the LBC in ERα (top) and ERβ (bottom).

A "mouse trap" model has been proposed (Moras*, et al.*, 1998) in which an agonist accesses the core of the LBD via a pore and once bound is "trapped" by a conformational shift of H12, a structural component of the LBD itself. This carboxyl-terminal α-helix folds against the surface of the LBD which is recognised by transcriptional coactivators that mediate the agonist-dependent transactivation properties of the ERs. The coactivator recognition surface or activation function (AF)-2 of the ER is created by α-helices 3, 4, 5 and 12 of the LBD and is composed of a hydrophobic groove that is capped on either side by two charged residues (charge clamp), a lysine residue from H3 and glutamate residue from H12 (Nolte*, et al.*, 1998,

Shiau*, et al.*, 1998). The coactivator motif that is recognised by this groove within the LBD is a conserved amphipathic α-helical structure with the consensus sequence LxxLL (L=leucine and x=any other amino acid) (Ding*, et al.*, 1998, Heery*, et al.*, 1997, LeDouarin*, et al.*, 1996, Savkur*, et al.*, 2004, Torchia*, et al.*, 1997). The recognition of the LxxLL motif by the LBD is facilitated by interactions between the hydrophobic side chains of the leucine amino acid residues displayed on one face of the co-activator protein with the hydrophobic surface of the LBD AF-2 site, and hydrogen bonding between the charge clamp residues at the AF-2 site and backbone carbonyl groups of the co-activator protein LxxLL motif (Nolte*, et al.*, 1998, Shiau*, et al.*, 1998). Thus, these ligand-regulated protein-protein interactions are crucial for the mediation of transcriptional activation by ERs (Wang*, et al.*, 2006).

6.1.2. Ligand Structure and ER Binding

Graham (2012) extensively reviewed the literature on the theory of ligand binding requirements, transcription, receptor protein structure and conformation and the applicability to computational docking. To summarise, there are a number of complex control mechanisms for estrogen target gene transcription, such as different expression of ER isoform combined with varying types of coregulatory proteins present in specific cell types. The molecular structures of endogenous estrogens are rigid and induce a precise 3D conformation in the ER upon binding. This precise conformation is observed in solved protein crystal structures and is described as the agonist conformation. This leads to distinct patterns in gene transcription. As mentioned in Section 1.3.1.4., pharmaceuticals such as tamoxifen, have been designed to block transcription by inducing the occupancy of the AF-2 site with H12 (Gangloff*, et al.*, 2001). Interestingly, genistein has similar properties to tamoxifen in ERβ (Barkhem*, et al.*, 1998), where the conformation adopted is antagonist in the absence of a coregulatory peptide (Pike*, et al.*, 1999); however, in the presence of this peptide, designed to mimic specific coregulatory proteins, ERβ adopts an agonist conformation. It is thought that the peptide is providing additional stabilisation necessary to produce the agonist conformation (Manas*, et al.*, 2004). Conversely in ERα, genistein induces an agonist conformation without the stabilisation of a co-regulatory peptide. This is likely because of the higher energy barrier between he agonist and antagonist conformation for ERα (Fig. 6.3); therefore, the energy barriers between these ER isoform conformations could be important when attempting to

predict the biological outcomes of xenoestrogen exposure cocktails (Gangloff*, et al.*, 2001, Shiau*, et al.*, 1998).

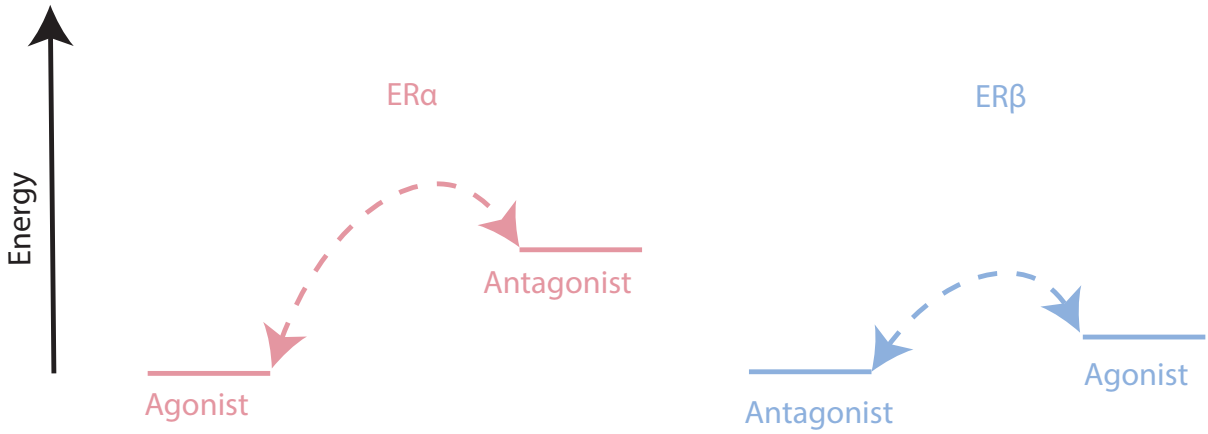


Figure 6.3: Illustration of proposed relative stability of the agonist and antagonist conformations of the two ERs (from Graham, 2012 with permission).

6.1.3. Requirements of Ligand Binding

The LBC requires key molecular characteristics of E2 to facilitate ligand interaction. This binding can be described as a 3D lock and key model, where only the correct key (i.e. ligand) will 'open' the lock (i.e. the ER). Therefore, it is known from the structure of E2 that two hydroxyl groups in the right spatial arrangement, one aromatic and the other aliphatic, separated by 9.6 Å of hydrophobicity are required to 'open' the ER. However, these requirements can be exploited by xenoestrogens which exhibit similar characteristics to E2; e.g. genistein has two aromatic hydroxyls separated by 13.13 Å of hydrophobicity. Ligands that more closely imitate E2 have greater estrogenic activity. Thus, this highly efficient and specific binding of ligands to the LBC are prerequisites for the desired ER-mediated biological activity. The specificity of these interactions is mediated by hydrogen bonds between key functional groups on the ER and on the ligands, namely Glu353/305, Arg394/346 and H_2O which forms a hydrogen bonded triumvirate with E2's aromatic hydroxyl, and His524/475 which hydrogen bonds with the aliphatic hydroxyl group of E2. Once the ligand is docked, hydrophobic interactions are created between the ligand and LBC amino acid residues pulling the α-helices towards the ligand and inducing ER conformational changes. These conformational changes are responsible for facilitating receptor dimerisation, nuclear translocation, and coactivator complex formation (e.g. ensuring the availability of the charge clamp residues at AF-2, see section 1.3.1.4.).

These specific interactions are also what is exploited in computational modelling when ligands are docked.

6.1.4. Computational Docking Background

Computational docking is the computer simulation used to predict binding of a ligand within the receptor binding cavity and to estimate the binding energy (affinity). It can be used for virtual screening of collections of potential drug candidates or screening a library of possible candidate chemicals, this approach is often utilised in pharmaceutical research (Knox*, et al.*, 2006, Knox*, et al.*, 2007, Knox*, et al.*, 2008, Sousa*, et al.*, 2006). Computational docking has been used extensively in the study of endocrine disrupting chemicals (EDCs), to enable an understanding of the specific interactions with the ER; for example, phytoestrogens (Lambrinidis*, et al.*, 2006), polychlorinated biphenyls, dichlorodiphyenyltrichloroethane (DDT) and its metabolites (D'Ursi*, et al.*, 2005), polyphenols (Lambrinidis*, et al.*, 2006), diphenyl ethers (Yang*, et al.*, 2010) and pesticides (Celik*, et al.*, 2008) have all been investigated using computational docking methods. However, most of the computational docking research has been applied to drug development for breast cancer, osteoporosis and prostate diseases (Knox*, et al.*, 2006, Knox*, et al.*, 2008).

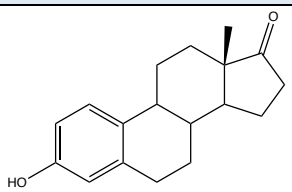
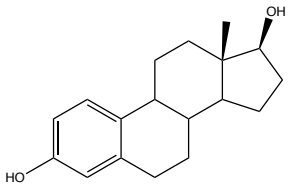
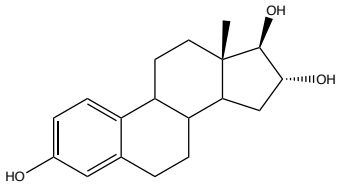
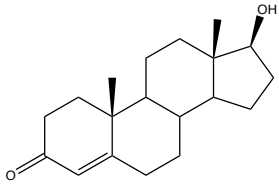
The basis of computational docking is the lock-and-key model first described by Fischer (Fischer, 1894). The 3D structures of the ligand and the receptor are both considered rigid and complement each other like a key fits into a lock (see Section 6.3.1.). It is based on the static models of the receptor and ligand, understanding how the ligand can adopt a shape that best complements and interacts with the binding cavity of the receptor. However, docking calculations do not consider the kinetic energy of the system, thus, large scale movements of the receptor that may occur as a result of ligand binding cannot be modelled using computational docking methods (Graham, 2012). Therefore, small-scale conformational changes in the protein structure in the vicinity of the binding cavity, termed flexibility, are now being considered in docking applications (Sousa*, et al.*, 2006). Thus, computational docking provides a means to study the intimate interactions of ligands with ERs without the high costs associated with other structural techniques such as x-ray crystallography.

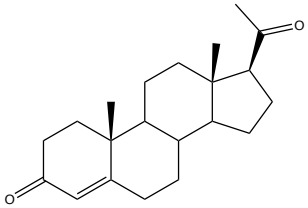
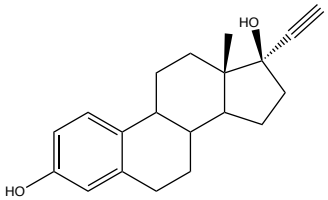
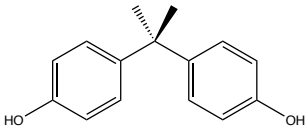
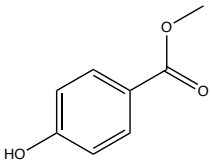
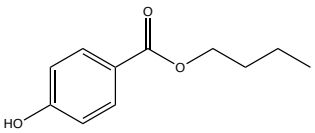
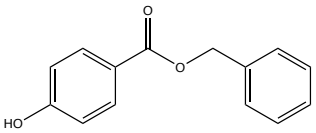
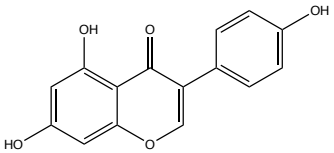
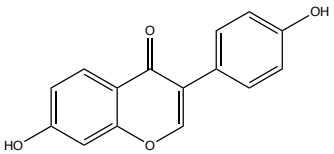
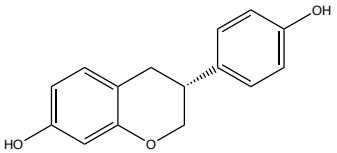
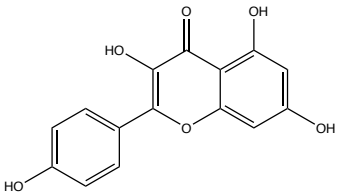
The aim of this study was to employ the established Schrödinger modelling platform to examine xenoestrogen binding at both LBC and AF-2 sites of both ER isoforms in order to determine potential binding affinities at both sites.

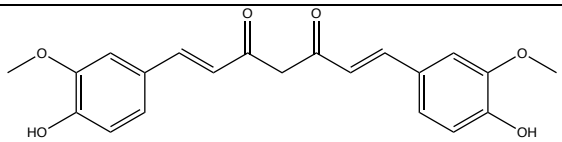
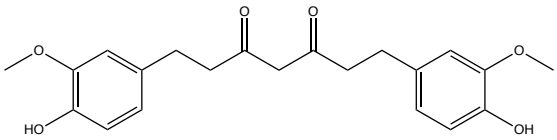
6.2. Experimental Approach

The xenoestrogens selected for this study include those already examined in Chapters 4 and 5, plus daidzein, equol, curcumin, benzylparaben, testosterone, progesterone and estrone (Table 6.1). These additional ligands were selected based upon relevance (e.g. naturally occurring steroid hormones) or persistence in the environment and foods. Each of the ligands were docked into each ER crystal structures: 1ERE and 3OLS. RRD and IFD docking methods were both used to investigate ER-ligand interactions.

Table 6.1: Structures of xenoestrogens selected for computational docking studies.

Ligand	Structure
Estrone	
E2	
Estriol	
Testosterone	

Progesterone	
EE2	
BPA	
Methylparaben	
Butylparaben	
Benzylparaben	
Genistein	
Daidzein	
Equol	
Kaempferol	

Curcumin	
Tetrahydrocurcumin	

The docking process begins with choosing or developing geometric and chemical descriptions of the separate receptor and small molecule structures. The receptor models are derived from the coordinates of ER x-ray crystal structures with co-crystallised ligands. The ligand model was developed using a 3D chemical structure drawing tool (2D sketcher, Maestro) (see Section 2.2.10.1). Energy minimisations of both receptors and ligands structures were carried out, and then using search algorithms and force field interactions, the docking process sought to find optimal placements of the ligands in the receptor binding cavity. A scoring function is used to calculate the energy of the receptor-ligand complex and ranks the poses of a given ligand relative to one another to allow the comparison of poses of different ligands (see Section 2.2.10.3). Ideally, the numerical value of the scoring function should correspond directly to the binding affinity of the ligand for the protein so that the ligands with the best scores are the best binders (Graham, 2012).

6.2.1. RRD and IFD Docking

Two docking methodologies were employed to study the xenoestrogen binding affinities, RRD and IFD. RRD is the simpler of the two methodologies, where the receptor cannot move so the degrees of freedom of the docking is that of the ligand: three translational, three global rotational and, one internal dihedral rotation for each rotatable bond (see Section 2.2.10.4). It was assumed that the ligand was able to adapt to the binding site to achieve a complex with favourable binding affinity (Fig. 6.4)

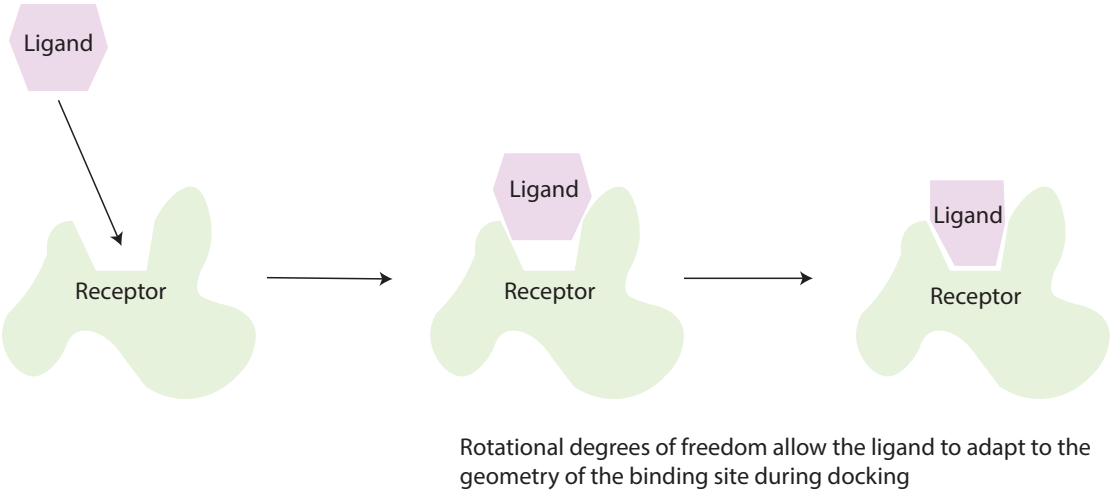


Figure 6.4: Illustration of the rigid receptor-flexible ligand docking concept (from Graham 2012 with permission).

On the other hand, it is well known that the ER experiences some degree of conformational change during the binding process, especially at the AF-2 site upon LBC binding (see Section 1.3.1.4.) (Zilli*, et al.*, 2009). Therefore, the xenoestrogens were also studied using IFD where different representations and simplifications of protein flexibility were implemented that avoid direct generation and search thousands of potential protein structure conformations (Graham, 2012). The Schrödinger platform uses an iterative approach of docking with a soft receptor followed by energy minimisation of the entire protein-ligand complex. An ensemble of candidate poses was generated per xenoestrogen within the receptor binding cavity. The candidate poses are then ranked, and a selection of the top-ranked poses were subjected to energy minimisation which allows the protein structure to relax around the ligand. These minimised receptor-ligand complexes were then re-scored to produce an ensemble of the most favourable complexes (see Section 2.2.10.6.).

6.2.2. Scoring Function

The scoring functions are used to estimate the free energy of binding (e.g. the binding affinity). A general scoring function was chosen because the objective of the study was to evaluate both multiple poses or clusters of similarity and the difference among the selected ligands, rather than being able to reliably use the information to refine the structure of the ligand to improve binding (Graham, 2012). Each of the scoring functions for each method, receptor and binding site, were compiled in empirical order. This was reported as the GlideScoreXP in units of kcal/mol and converted to kJ/mol where 1 kcal/mol = 4.2 kJ/mol (Bash*, et al.*, 1996, Mulholland, 2007)

6.3. Results

Several ligands were used to dock at both LBC and AF-2 sites in both ER isoforms, ERα and ERβ, in order to validate binding and capture subtle variations caused by the different ligands. A total of two models were developed and two docking processes were used to evaluate binding. Rigid docking holds the models' static, and IFD allows the models to mutually adapt. With rigid docking, the subtleties among the different models are retained and the docking calculation determines how favourably the particular ligand interacts with a given model. Thus, for less flexible ligands, the ligands were only docked into models that they were able to fit. Poor fitting was evident for less favourable calculated binding energies. More flexible the ligands were able to adapt to the fixed binding topography of the binding site compared to the rigid ligands. The calculated binding energies indicate how 'uncomfortable' a particular binding site is for a particular ligand. Among the flexible ligands, the relative differences in calculated binding energies indicate which of the different receptor-ligand complex conformation is more favourable. In contrast, IFD allowed the receptor model to adapt to a ligand pose and one would expect similar models to converge to a common conformation for a given ligand, if the ligand is effective in influencing the receptor conformation.

6.3.1. RRD and IFD Validation

The first step in the studies was to validate both the RRD and IFD docking methods. The ability of the docking procedure to correctly place the ligand and correctly predict the binding energy was evaluated in both the 1ERE and 3OLS models. Graham (2012) extensively validated both docking methods, comparing the calculated binding energies with experimental binding energies from the literature. Thus, correlations between binding energies of ligands from Graham's studies (E2, genistein and methylparaben) were compared to those generated in the current study in order to validate the methods used in the studies. The results showed a strong correlation between the RRD and IFD docking scores (Fig. 6.5) with R^2 values of 0.98 for ERα and 0.95 for ERβ, in both RRD and IFD.

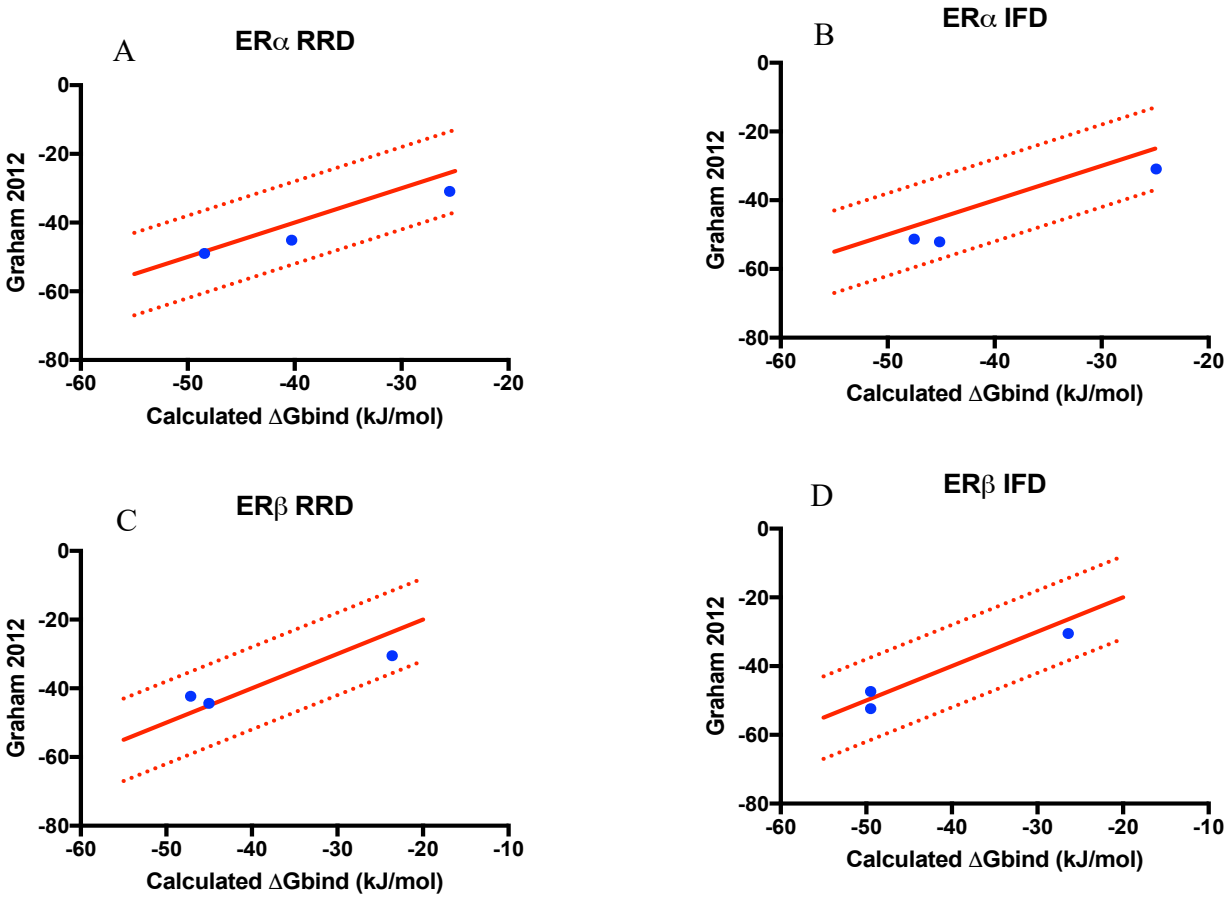


Figure 6.5: Correlation of Graham (2012) calculated binding energies and calculated binding energies for both RRD (a and c) and IFD (b and d) in both ERα and ERβ models, 1ERE and 3OLS, respectively.

6.3.2. LBC Docking Studies

The calculated RRD binding energies for the top ranked poses for both ERα and ERβ LBCs are given in Table 6.2. RRD was able to dock all the ligands into the correct pose in both ERα and ERβ models. IFD had the same success for docking the ligands into both ER models.

Table 6.2: Calculated binding energies (kJ/mol) for the top-ranked poses for LBC obtained by RRD and IFD in both ERα (PDB: 1ERE) and ERβ (PDB: 3OLS).

	ERα binding energies (kJ/mol)		ERβ binding energies (kJ/mol)	
	RRD	IFD	RRD	IFD
Estrone	-35.4	-44.8	-44.9	-46.2
E2	-48.4	-47.5	-47.2	-49.5
Estriol	-50.2	-51.7	-50.7	-54.5
Testosterone	-43.5	-46.	-42.4	-45.7
Progesterone	-22.0	-40.5	-35.5	-47.8
EE2	-41.4	-51.7	-41.2	-50.2
BPA	-37.1	-37.9	-42.2	-40.0
Methylparaben	-25.5	-24.9	-23.6	-26.4
Butylparaben	-29.8	-31.5	-28.5	-29.2
Benzylparaben	-37.4	-40.7	-37.0	-41.0
Genistein	-40.3	-45.2	-45.0	-49.5
Daidzein	-31.8	-42.	-35.9	-47.0
Equol	-39.5	-45.21	-43.4	-48.2
Kaempferol	-42.7	-48.0	-41.2	-50.2
Curcumin	-31.4	-55.7	-38.2	-57.7
Tetrahydrocurcumin	-51.5	-53.3	-40.4	-49.0

Correlation of the RRD and IFD calculated binding energies are shown in Figure 6.6. $R^2 = 0.41$ for ERα and 0.57 for ERβ. Although both R^2 values show a weak correlation, the P values of 0.0077 and 0.0007 respectively indicate a significant correlation for ERα and ERβ models.

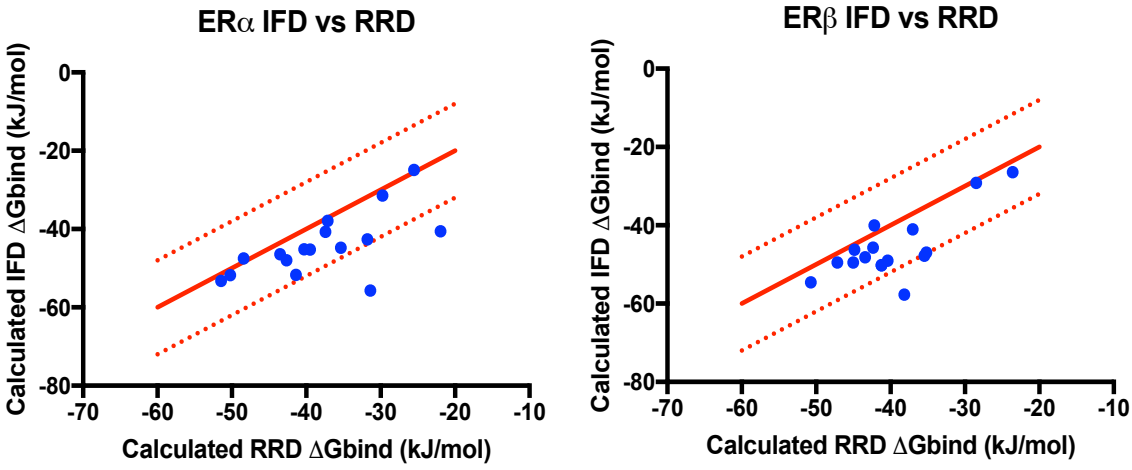


Figure 6.6: Correlation of RRD calculated binding energies and IFD calculated binding energies in ERα and ERβ models, 1ERE (left) and 3OLS (right).

To further validate the results, correlations between the calculated binding energies with experimental ΔG_{bind} (Table Appendix 4) are shown in Figure 6.7 for both RRD and IFD ERα and ERβ models. Both RRD and IFD predict the experimental binding energies for these ligands within the stated uncertainty of ±12.5 kJ/mol. There are only two ligands that were not docked within the stated uncertainty, testosterone and progesterone.

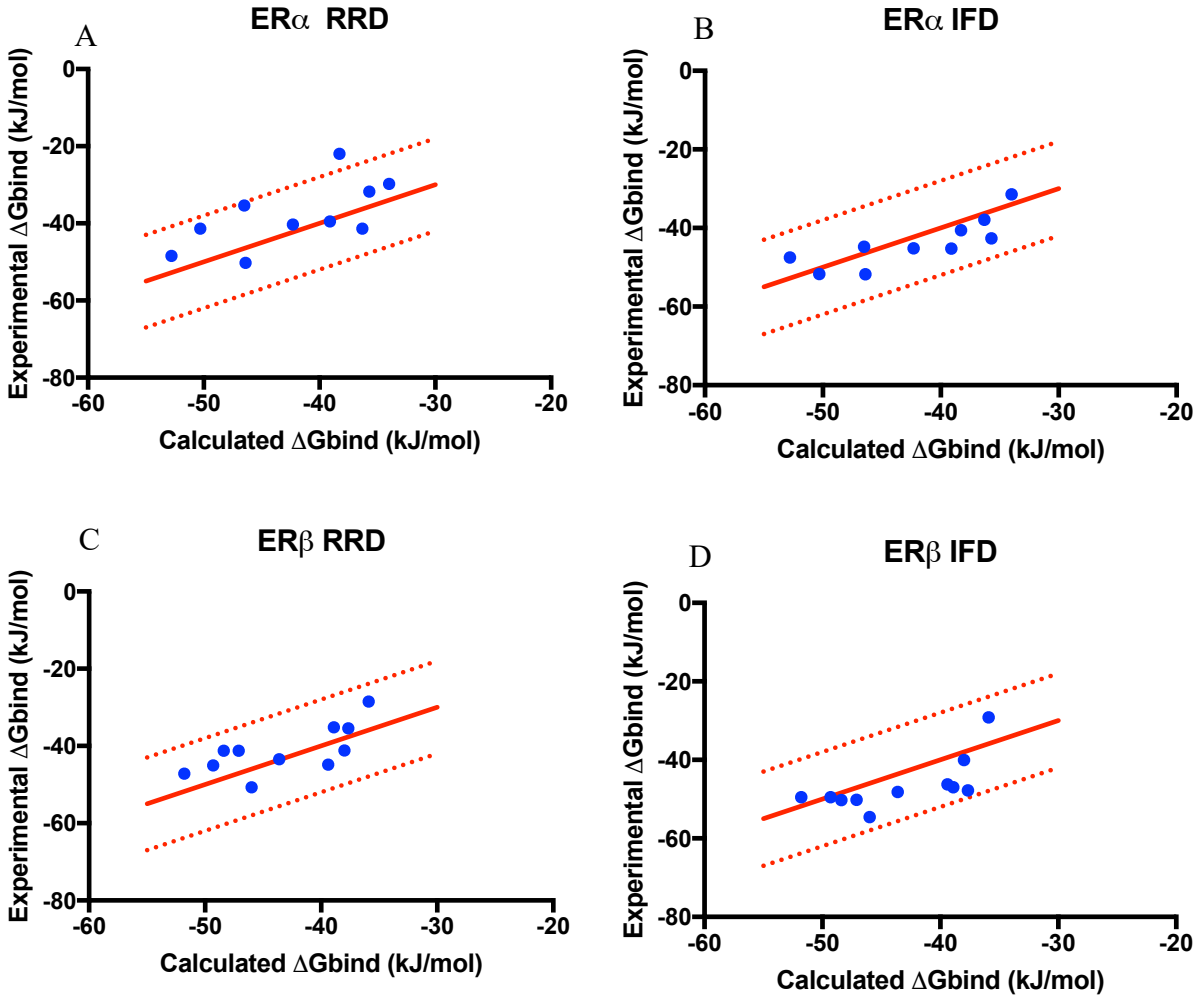


Figure 6.7: Correlation of experimental and calculated binding energies for RRD (a and c) and IFD (b and d). Solid line indicates 1:1 correlation; broken lines indicate stated uncertainty of ± 12.5 kJ/mol.

Methylparaben, benzylparaben, curcumin and tetrahydrocurcumin do not have literature values for experimental binding affinities kaempferol and testosterone have only been studied in ERβ and ERα, respectively. Therefore, data were not included if there were no experimental data to determine the correlation.

Furthermore, there are significant differences in potencies between some xenoestrogens for RRD and IFD docking methodologies (Table 6.3). For example, estradiol and tetrahydrocurcumin have an RRD ERα/ERβ ratio of 1.03 and 1.27, respectively, showing that they bind preferentially to ERα LBC using RRD docking. On the other hand, genistein and daidzein have RRD ERα/ERβ ratios of 0.89 and 0.9, respectively, showing that they preferentially bind to ERβ LBC. Interestingly, IFD appears to reduce the variability of the ERα/ERβ ratios showing ligands to have lower ER isoform binding preference.

Table 6.3: RRD and IFD ER isoform preference from calculated binding energies (kJ/mol).

Ligand	RRD preference ratio ERα/ERβ	IFD preference ratio ERα/ERβ
Estrone	0.8	1.0
E2	1.0	1.0
Estriol	1.0	1.0
Testosterone	1.0	1.0
Progesterone	0.6	0.9
EE2	1.0	1.0
BPA	0.9	1.0
Methylparaben	1.1	1.0
Butylparaben	1.0	1.1
Benzylparaben	1.0	1.0
Genistein	0.9	0.9
Daidzein	0.9	0.9
Equol	0.9	0.9
Kaempferol	1.0	1.0
Curcumin	0.8	1.0
Tetrahydrocurcumin	1.3	1.1

6.3.3. AF-2 Docking Studies

The calculated binding energies from RRD ligand binding studies at ERα and ERβ AF-2 are summarised in Table 6.4. Literature values for experimental binding affinities for some ligands were not available; therefore, the most favourable binding energy when docked is shown. Some ligands were not successfully docked using RRD in the ERα model, these included estrone, testosterone, methylparaben, butylparaben, benzylparaben, genistein, daidzein and equol. In addition, no docked ligands were returned for IFD docking studies in the 1ERE and 3OLS models. Instead, the Schrödinger modelling platform docked the ligands at the more favourable LBC binding site. This was also observed with the ligands that were not docked in the RRD ERα model.

Table 6.4: Calculated binding energies (kJ/mol) for the top-ranked poses for AF-2 obtained by RRD in both ERα (PDB: 1ERE) and ERβ (PDB: 3OLS). ND=not docked and indicates the ligand was not docked in the ER models.

	AF-2 RRD binding energies (kJ/mol)	
Ligand	ERα	ERβ
Estrone	ND	-7.0
E2	-6.6	-14.6
Estriol	-13.6	-13.8
Testosterone	ND	-10.4
Progesterone	-10.0	-12.4
EE2	-5.6	-7.7
BPA	-10.1	-10.1
Methylparaben	ND	-11.9
Butylparaben	ND	-12.00
Benzylparaben	ND	-10.9
Genistein	ND	-11.8
Daidzein	ND	-11.0
Equol	ND	-10.5
Kaempferol	-14.4	-12.4
Curcumin	-14.8	-10.2
Tetrahydrocurcumin	-16.2	-10.7

Interestingly, the docking results showed there were clear key amino acid interactions at the AF-2 that were consistent between both ERα and ERβ models (e.g. with charge clamp amino acid residues). For many of the docked ligands, either a hydrogen bond or van der Waals interaction was formed at Gln375/327. In addition, most of the docked ligands formed either hydrogen bond or van der Waals interaction with at least one of the charge clamp residues (Lys362/314 and Glu542/493). Other consistent van der Waals interactions that emerged from the AF-2 docking studies

were observed with amino acid residues Met358/309, Leu372/324, Val376/328, Leu379/331 and Leu539/490. Interactions of ERβ ligands with AF-2 amino acid residues also included Ile310, Phe319, Glu332 and Met494; however, these interactions were not observed in ERα AF-2 docking studies. Example data for tetrahydrocurcumin and curcumin are shown in Figure 6.8 which highlights hydrogen bonding interactions for both ER isoforms.

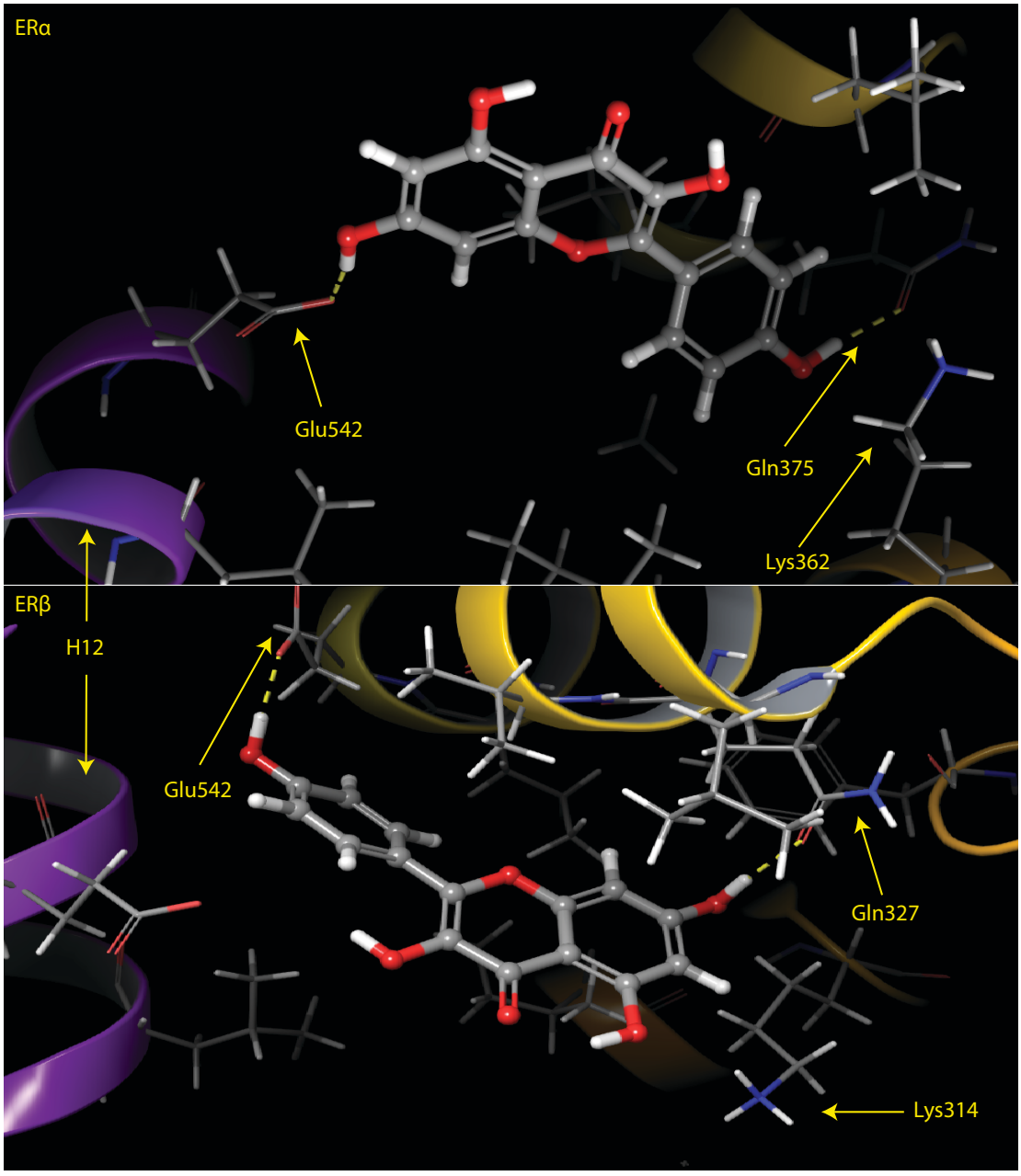


Figure 6.8: Hydrogen bonding interactions at the AF-2 site with tetrahydrocurcumin (ERα; top) and curcumin (ERβ;bottom).

Surprisingly, some ligands (i.e. kaempferol and tetrahydrocurcumin) exhibited hydrogen bonding interactions between both charge clamp amino acid residues (i.e. Lys362/314 and Glu542/493) and the OH moieties on the ligands, imitating co-activator interactions at the AF-2 site. Example data are shown for kaempferol (ERα) and tetrahydrocurcumin (ERβ) in Figure 6.9.

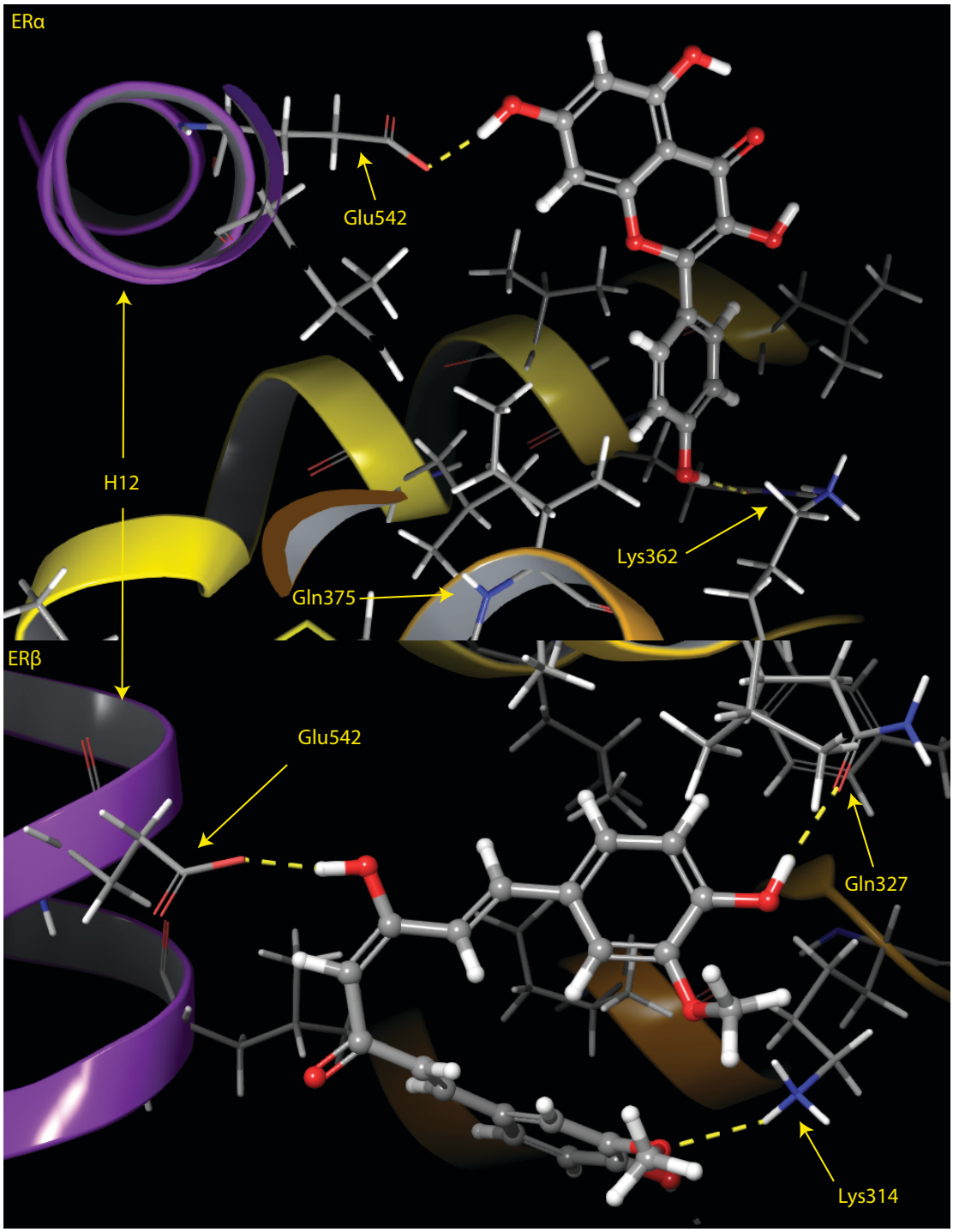


Figure 6.9: Hydrogen bonding interactions with kaempferol (ERα; top) and tetrahydrocurcumin (ERβ; bottom) with the charge clamp amino acid residues at the AF-2 site.

6.3.4. LBC and AF-2 Binding Preference

Comparisons between the LBC and AF-2 calculated binding energies demonstrate ligand binding is significantly favoured towards the LBC. This suggests that the LBC is a high affinity binding site and the AF-2 is a lower affinity binding site. The ratios show that some ligands (i.e. E2 and EE2) have much greater LBC favourability (i.e. 7.4 times more favourable) compared to other ligands (e.g. kaempferol or curcumin) where the LBC binding affinity is only between 2 and 3 times more favoured. These ratios are summarised in Table 6.5.

Table 6.5: ERα and ERβ binding site preference ratio (LBC/AF-2). ND = not docked and indicates the ligand was not docked in the model.

Ligand	ERα preference ratio (LBC/AF-2)	ERβ preference ratio (LBC/AF-2)
Estrone	ND	6.5
E2	7.4	3.2
Estriol	3.7	3.7
Testosterone	ND	4.1
Progesterone	2.2	2.9
EE2	7.4	5.4
BPA	3.7	4.2
Methylparaben	ND	2.0
Butylparaben	ND	2.4
Benzylparaben	ND	3.4
Genistein	ND	3.8
Daidzein	ND	3.2
Equol	ND	4.1
Kaempferol	3.0	3.3
Curcumin	2.1	3.7
Tetrahydrocurcumin	3.2	3.8

6.4 Discussion

6.4.1. Limitations

Although there is a good theoretical and physical basis for the calculations in computational docking, the complexity of the systems necessitates assumptions and simplifications in order to make the problem more tractable and computationally possible with our current computer systems. While more sophisticated systems are available they are incredibly expensive; therefore, it was not possible to gain access to these systems for these studies. For example, the Schrödinger modelling platform assumes that only the amino acid residues within 20 Å of the docking site are able to

move. The assumptions and simplifications can result in errors and disagreement between computational results. For the present study, the scoring function implemented in the Schrödinger Suite is a good choice because ERα is one of the systems used (Friesner*, et al.*, 2004, Friesner*, et al.*, 2006, Halgren*, et al.*, 2004) in the calibration of the *GlideScore* scoring function and validation of the *Glide* docking methodology. *Glide* has been shown to perform better than many other docking software packages in both accuracy of predicted binding energies and in accuracy of ligand placement (Friesner*, et al.*, 2004, Friesner*, et al.*, 2006, Halgren*, et al.*, 2004). Therefore, it is not unreasonable to expect that the calculated binding energies and ligand poses obtained from the Schrödinger Suite for this study of ERα and ERβ are likely to be representative of the *in vivo* situation. However, for the purposes of this work, it is still important to recognise that computational results may not always agree numerically with experimental results but should reflect the trends demonstrated by experimental results. For these reasons, validation of the model results using known test scenarios is essential to gain confidence in the computations for predicting behaviour of unknown systems (Graham, 2012).

With the RRD receptor model, the binding cavity residues are held fixed and the ligand is required to adapt to the binding cavity to find a low energy conformation. Therefore, the positions of the key binding cavity amino acid residues are important in determining ligand positioning within the binding cavity because of the corresponding interacting moieties on the ligand and in the binding cleft. When performing IFD, it is reasonable to expect that any given ligand would be docked into any of the sites on the models, provided that the adjustment of the models by the induced-fit procedure allows the model to adapt to the ligand. However, in the case of AF-2 docking the induced-fit procedure grappled with the less definitive site and facilitated ligand docking to the more specific, defined site of the LBC; thus, IFD was not the docking method of choice when attempting to dock ligands at the AF-2 sites of the models.

6.4.2. LBC Binding Studies

The validation of the LBC docking demonstrated that:

- Both RRD and IFD can correctly place the ligands into the models and place the ligands in known orientations within the binding cavity of the models

- The scoring function can calculate the experimental binding energy within the stated uncertainty of ± 12 kJ/mol.

There were good correlations between the calculated LBC binding affinities using both RRD and IFD for both the ERα and ERβ models. Correlations were also found between calculated binding affinities and experimental binding data from the literature (Table A1). Interestingly, the IFD returned an overall stronger correlation compared to RRD for both ER models. However, the difference in correlation was not significant. This suggests that either of the protocols can be used to calculate ER ligand binding affinity. For some of the ligands it was not possible to find experimental binding affinities; however, due to the good correlation between calculated and experimental binding energies it is likely the LBC binding data is representative of those ligands for ERα and ERβ.

Surprisingly, estriol and tetrahydrocurcumin returned the best binding affinities for ERα. This was unexpected as EE2 is usually the superior binder; however, docking returned a binding affinity of -41.39 kJ/mol using RRD compared to -50.21 kJ/mol and -51.45 kJ/mol for estriol and tetrahydrocurcumin, respectively. RRD was unable to reliably dock EE2 at the LBC, with a lack of His524 hydrogen bond formation. This was surprising because EE2 is a pharmaceutical designed to interact with key elements of the LBC. This likely explains the lower calculated binding affinity (Fig. 6.10). In comparison, estriol had the highest binding affinity (-50.70 kJ/mol) followed by E2 (-47.15 kJ/mol) and genistein (-45.01 kJ/mol) in the RRD ERβ model. In general, the IFD method returned higher binding affinities compared to RRD. This is likely due to the ER protein models being 'softened' allowing the model to adapt to the ligand rather than the ligand having to adapt to the rigid protein. In short, in IFD receptor plasticity and ligand plasticity allow for the mutual adaptation of both the receptor and ligand facilitating optimum binding of the ligand to the binding site. However, it does not indicate the reliability of the model to represent the *in vivo* situation. Thus, a greater binding affinity does not necessarily correlate to better *in vivo* accuracy. It is clear from the ERα/ERβ ratios that IFD does not show the correct ER isoform preference for ligands such as E2; therefore, RRD likely more closely represents *in vivo* binding affinities.

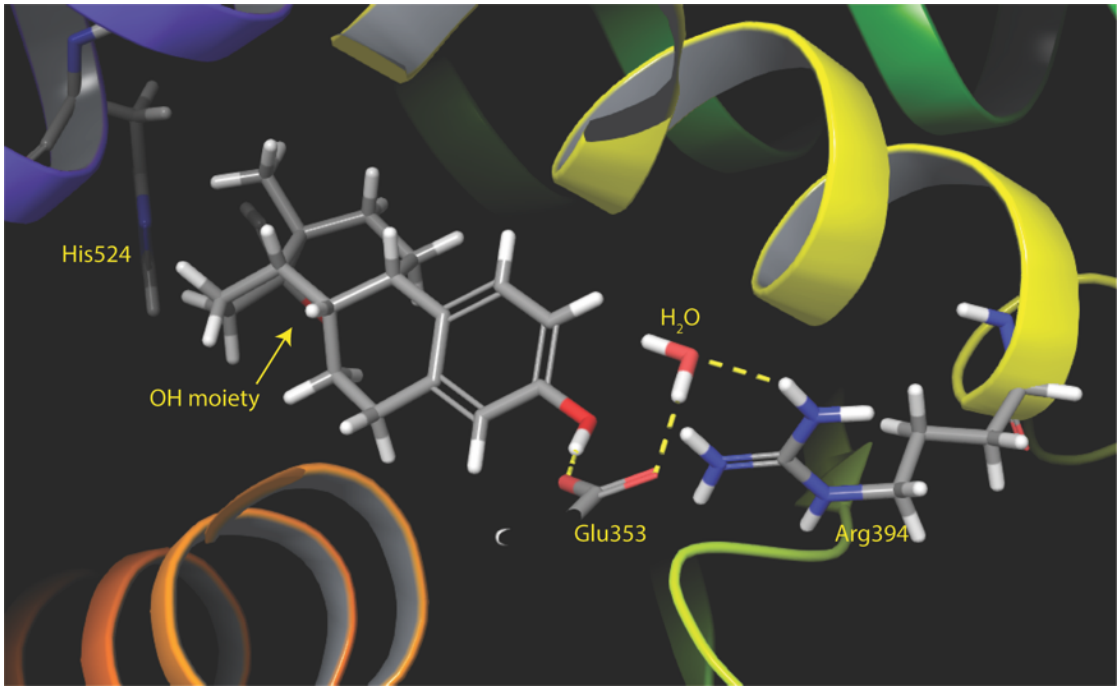


Figure 6.10: LBC docking of EE2, illustrating the absence of hydrogen bonding between the aliphatic hydroxyl and His524 in ERα.

6.4.3. AF-2 Binding Studies

Ligands were successfully docked at the AF-2 site in both ER models using RRD; however, in the ERα model not all ligands were successfully docked compared to ERβ where all ligands were docked. For the first time this provides evidence for xenoestrogen interactions at the AF-2 site. Interestingly, when comparing the docking results presented in this chapter with the study by Wang *et al*., (2006), which reports studies on 4-OHT co-crystallised at the ERβ AF-2 site - the amino acid interactions they showed are strikingly similar to those seen in the present studies. For example, Wang and colleagues found the AF-2 interactions to be mainly hydrophobic or van der Waals interactions which were emulated in the ERβ docking studies (Wang*, et al.*, 2006). In addition, the amino acid residues involved in ligand interactions shown in the present study are very similar to those described by Singh *et al.,* (2015) (Table 6.6). Interestingly, the ligands (candidate development drugs) studied by Singh *et al.,* (2015) had significant structural similarities to curcumin (i.e. one of the strongest AF-2 binders in the present studies). This agreement between data between different groups at different times strongly support the proposed xenoestrogen AF-2 interactions. This suggests that the AF-2 interactions presented in this chapter could also be occurring *in vivo*.

Table 6.6: AF-2 amino acid residue studies presented in this thesis comparisons between docking studies and published AF-2 interactions in ERα and ERβ. The tick indicates that the amino acid residue was involved in the ligand interaction with AF-2.

ER AF-2 amino acids (ERα/ERβ)	Thesis studies	Wang and colleagues	Singh and colleagues
Leu354/306		✓	
Met358/309	✓	✓	✓
Ile359/310	✓	✓	✓
Lys362/314	✓	✓	✓
Phe367/319	✓		
Leu372/324	✓		✓
Gln375/327	✓	✓	✓
Val376/328	✓	✓	✓
Leu379/331	✓	✓	
Glu380/332	✓	✓	
Trp383/335		✓	
Leu496/447		✓	
Arg497/448		✓	
Leu539/490		✓	
Glu542/493	✓	✓	✓
Met543/494	✓		✓
Met546/497		✓	

6.4.3.1. Interactions with Gln375/327

It is clear that particular amino acids play key roles in AF-2 docking as seen in Table 6.6. Interestingly, Gln375/327 has previously been found to play an important role in stabilisation of AF-2 binding (Singh*, et al.*, 2015). Singh and colleagues found that the ligands that exhibited weak antagonist effects in cultured MCF-7 breast cancer cell exposure studies did not interact with Gln375, highlighting the importance of ligand interactions with this amino acid. Furthermore, all the ligands that were docked at the AF-2 site either had a hydrogen bond or a strong van der Waals interaction with Gln375/327 highlighting its possible importance in facilitating AF-2 site binding. Therefore, Gln375/327 is likely a key amino acid involved in small ligand (e.g. xenoestrogens) interactions at the AF-2 site via the formation of either a hydrogen bond or van der Waals interaction.

6.4.3.2. Charge Clamp Amino Acid Residue Interactions

In addition to the importance of a hydrogen bond or van der Waals interaction with Gln365/327, the charge clamp amino acid residues Lys362/314 and Glu542/493, which are known to facilitate key interactions between the coregulatory protein LxxLL binding motif and the AF-2 site, are also key in facilitating ligand docking in

this study. A majority of ligands that were docked at AF-2 in both ERα and ERβ model had at least one strong interaction with these charge clamp residues, with some ligands (e.g. curcumin and tetrahydrocurcumin) hydrogen bonding at both charge clamp amino acid residues. While these amino acid residues are usually responsible for recognition of the coactivator LxxLL motif, it is likely that the hydrogen bonding interactions between the charge clamp amino acid residues and the OH moieties on the ligands are anchoring ligand binding, facilitating the stabilisation of the ER-ligand complex (see Fig. 6.7). This stabilisation induced by AF-2 binding is likely important in disrupting the co-activator-DNA complex formation, which could affect ER-mediated gene transcription (see Chapter 4). Indeed, the studies by Singh *et al.* (2015) demonstrated that the interactions between charge clamp amino acid residues and the ligand was important in facilitating a more potent anti-proliferative effect in breast cancer cell lines. Therefore, understanding interactions between the AF-2 site and xenoestrogens could be important in determining the anti-proliferative effects which could be used to treat and reduce breast cancer risk, this will be discussed further in Section 6.4.5.

6.4.3.3. Additional AF-2 Amino Acid Interactions

There are a number of other amino acid residues that are consistent with previous studies, namely Met358/309, Leu372/324, Val376/328, Leu379/331 and Leu539/490 in both ERα and ERβ models. Additional interactions with ERβ included Ile310, Phe319, Glu332 and Met494 which were consistent with the findings of Wang *et al.* (2006) and Singh *et al*., (2015). The example of kaempferol bound at ERα AF-2 is shown in Figure 6.11. The way a molecule interacts with the ER can determine its biological activity (e.g. in terms of agonism or antagonism); therefore, some xenoestrogens could facilitate their mixed agonist/antagonist effects via a two-site binding model. The two-site binding model, previously discussed in Chapters 4 and 5, is demonstrated by the results reported here, where the LBC is the primary, high affinity binding site and the AF-2 is the secondary, low affinity binding site. Thus, the mixed agonist/antagonist effects could be explained by the two-site binding model. In addition, some xenoestrogens exhibited similar binding characteristics to the ligands investigated by Singh and colleagues; therefore, it is reasonable to assume that some xenoestrogens, which were also shown in this thesis to have ER-mediated anti-proliferative effects, are probably facilitating anti-proliferative effects in breast cancer cells via binding to the AF-2 site. The logical downstream effects of binding, as

discussed in Chapters 4 and 5, could interfere with ER-mediated transcription and thus gene expression and ER-mediated cellular signalling pathways (e.g. cell cycle and apoptosis). Therefore, understanding the mechanism of action of mixed agonist/antagonist effects and the biological implications for exposures to xenoestrogens combinations is important in understanding xenoestrogens exposure cocktails as breast cancer risk factors.

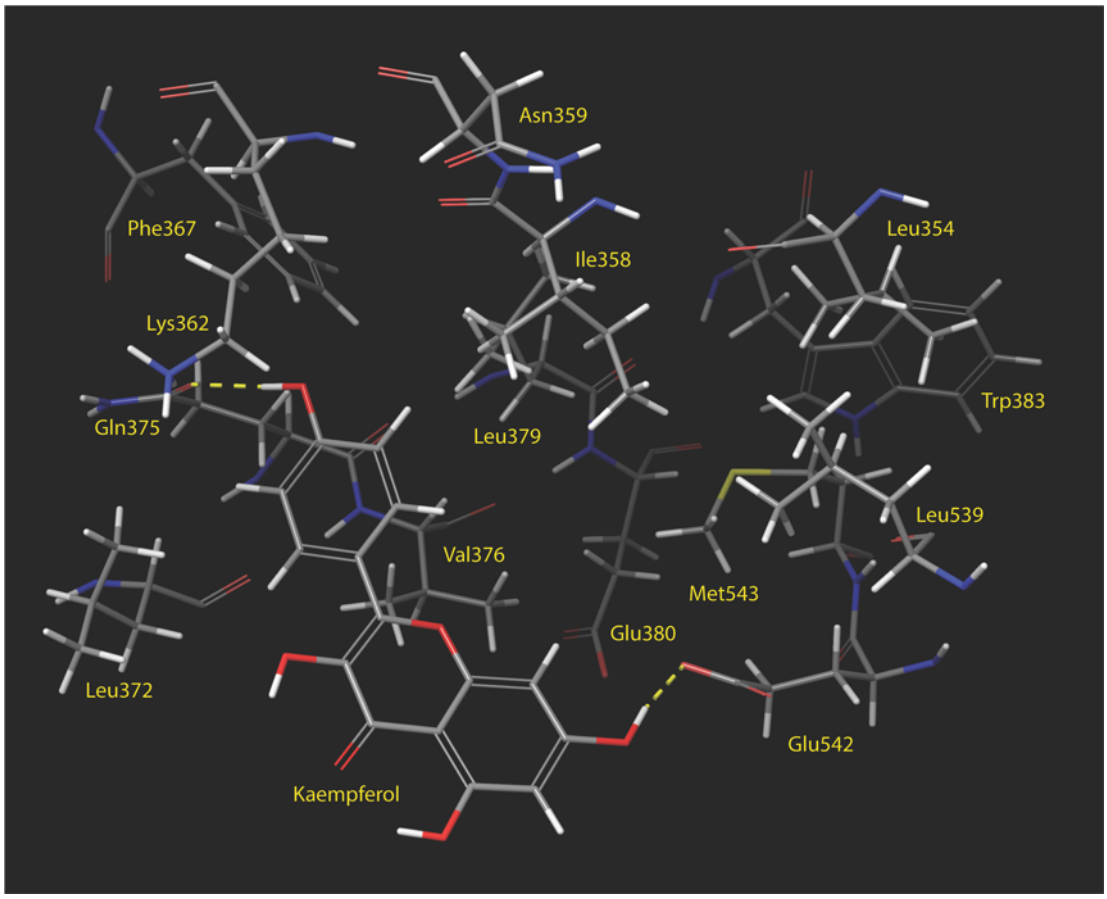


Figure 6.11: Key amino acid residue interactions with kaempferol at the ERα AF-2 site.

6.4.3.4. AF-2 Binding Energies

Tetrahydrocurcumin, estriol, kaempferol and curcumin had the highest calculated binding energies for AF-2 in the ERα model, while E2, estriol, kaempferol and progesterone had the highest calculated binding energies for AF-2 in the ERβ model. Interestingly, the ligands that had the highest AF-2 calculated binding energies in the ERα models, were also the ligands that had the greatest anti-proliferative effects in MCF-7 cell studies (see Chapter 4). This is not surprising given the high expression of ERα in MCF-7 cells (Al-Bader, *et al.*, 2011); therefore, a correlation between AF-2 binding energies and MCF-7 cell studies was not surprising.

Whilst all ligands were docked in the ERβ model, only some of the ligands were successfully docked in the ERα model using RRD. The main difference between the AF-2 sites of the ERα and ERβ models is that ERβ is co-crystallised with a co-regulatory peptide while ERα is not. As discussed above, ERβ has a lower energy barrier between the agonist and antagonist conformations compared to ERα; therefore, ERβ requires a coactivator peptide to provide stabilisation of the agonist conformation. Upon closer examination of the key AF-2 amino acid residues, it is clear ERβ's coactivator peptide influences the distances between the key AF-2 site residues (i.e. Lysine, glutamate and glutamine) (Fig. 6.12). While the differences between the amino acid residues is small (i.e. < 1 Å) between ERα and ERβ models, it appears to be sufficient to differentiate between a docked and 'not docked' ligand. Although this is not the only reason for influencing ligand docking at AF-2, it is likely to be one of the major contributing factors.

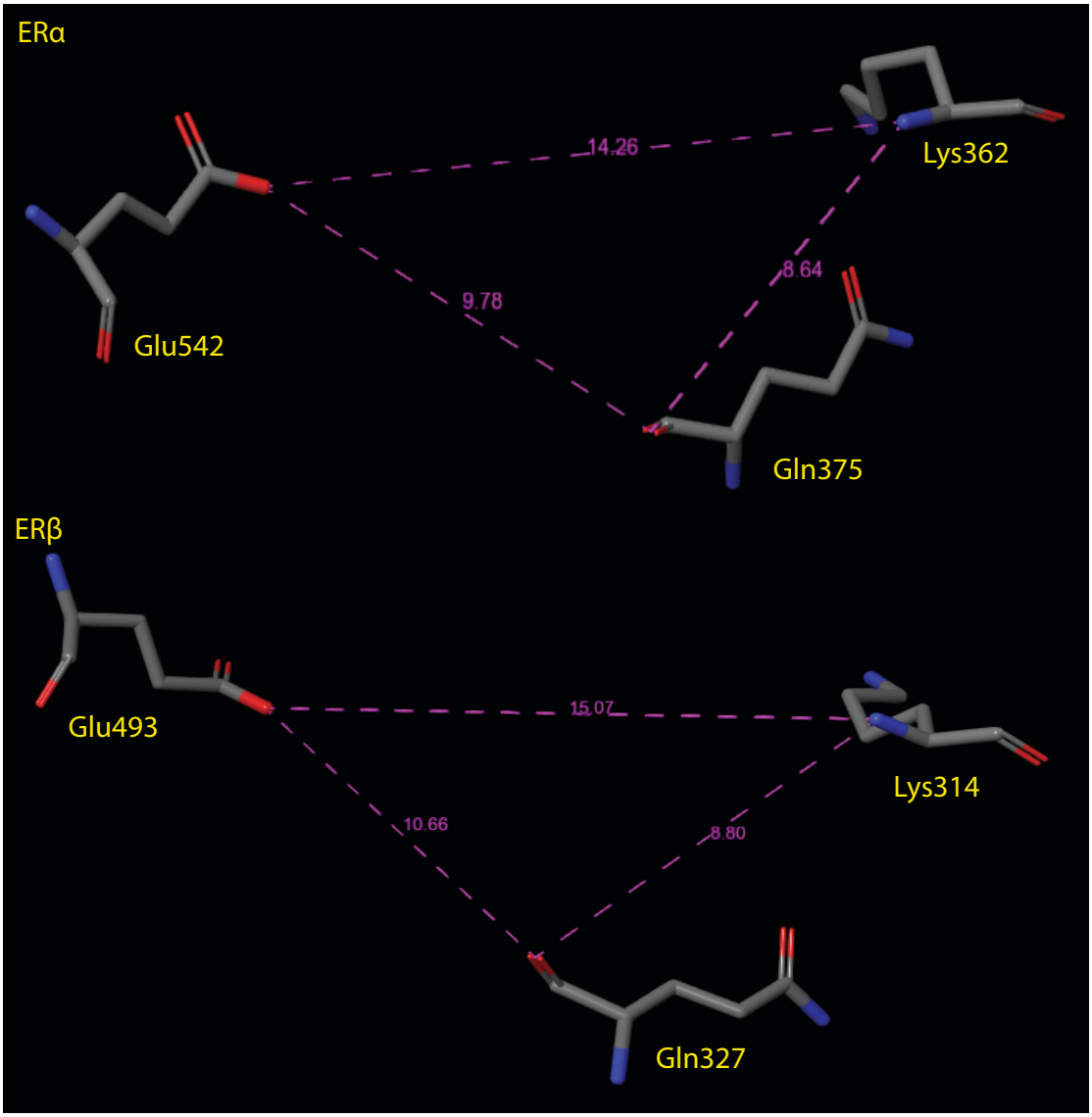


Figure 6.12: Distances (Å) between the key AF-2 amino acid residues Lys362/314, Gln375/327 and Glu542/493 in ERα (top) and ERβ (bottom) models. This outlines the differences between the key amino acid residues of the models.

In addition to AF-2 amino acid residues' positions, the length and flexibility of a ligand (NB this is RRD docking) may also influence ligand docking at AF-2. Comparisons between key ligand moieties (e.g. hydroxyl or carbonyl groups) prior and post docking illustrate how a ligand might adapt to the AF-2 site in the docking process (Table 6.7). The ability of a ligand to adapt to the AF-2 site could influence the binding and even the strength of binding (i.e. binding energy); thus, an optimum distance between ligand moieties of 9-12 Å appears to result in docking to the ER AF-2 site. Furthermore, the positioning of the key amino acid residues (see above) may contribute to the ligand's ability to best adapt to the AF-2 site. For example, the

separation of key kaempferol hydroxyls is 12.2 Å prior to docking and reduced to 11.1 Å post docking, clearly indicating the ability of the ligand to adapt to the optimum AF-2 binding separation (i.e. 9-12 Å). However, some ligands did not have sufficient plasticity to adapt to the docking requirements of the ERα AF-2 site. For example, genistein which has a hydroxyl separation of 13.1 Å prior to docking, could not be docked in the ERα model but was successfully docked in the ERβ model. This suggests that the combination of a greater distance between amino acid residues and greater genistein hydroxyl separation prohibited its docking in the ERα model.

In addition, ligand rigidity is likely to influence the ability of a ligand to be docked at AF-2. RRD utilises a rigid receptor with a flexible ligand, where the ligand has to adapt to the rigid binding site requirements for AF-2. Therefore, some ligands which have a greater moiety separation (e.g. curcumin) were docked in preference to ligands that had smaller separation (e.g. genistein) because of their greater molecular flexibility. Curcumin has greater flexibility compared to genistein, with a key moiety separation of 9.09 Å post docking. This is well within the ideal range of separation for binding to the AF-2 site. In view of this, it is not surprising that curcumin was docked and ligands such as genistein and daidzein were not. On the other hand, while RRD had limited success docking ligands in the ERα model, IFD was not successful in docking any of the ligands in both ERα and ERβ models. Whilst receptor flexibility is likely more representative of the *in vivo* situation compared to RRD, the AF-2 site is a much broader, less defined binding site. Therefore, using the Schrödinger platform it was not possible to determine a favourable docking position at AF-2 for some ligands (e.g. genistein), which results in them being docked in the more defined and specific LBC.

Table 6.7: Ligand moiety separation prior and post docking in the Schrödinger computer modelling platform.

Ligand	Ligand separation prior to docking (Å)	ERα Ligand separation post docking (Å)	ERβ Ligand separation post docking (Å)
E2	10.2	11.9	11.1
Estrone	11.0		11.4
Estriol	11.5	10.5	11.1
EE2	10.1	9.7	9.9
Testosterone	10.1		10.6
Progesterone	10.2	10.1	8.7
Genistein	13.1		10.0
Daidzein	13.1		11.3
Kaempferol	12.2	11.1	11.1
Equol	8.9		12.4
Curcumin	13.5	9.1	8.5
Tetrahydrocurcumin	12.2	9.7	7.4
Methylparaben	8.5		8.5
Butylparaben	12.6		9.7
Benzylparaben	12.2		10.7
BPA	10.4	10.5	10.1

6.4.4. LBC/AF-2 Binding Affinity Ratios

The LBC/AF-2 ratios (Table 6.5) indicate the favourability of ligands for LBC compared to AF-2. The ligands can be grouped according to their LBC/AF-2 ratios (i.e. high LBC binding affinity and low AF-2 binding affinity) and those that have a low ratio (i.e. lower LBC binding affinity and low AF-2 binding affinity). For example, E2 in the ERα model has a high LBC/AF-2 ratio of 7.4 compared to estriol and tetrahydrocurcumin which have ratios of 3.7 and 3.2, respectively. This might find use in the prediction of the biological effects of estrogen mimic cocktails. For example, a mixture containing a high ratio ligand and a low ratio ligand could have a different effect when compared to a mixture with two low or two high ratio ligands. Thus, the lower the LBC/AF-2 ratio the more likely the ligand is to favour AF-2 binding which could result in anti-proliferative effects. However, the LBC/AF-2 is still dependent on the individual binding affinities; for example, if the ligand had a low binding affinity to AF-2, the chance of it inducing an anti-proliferative affect would be low because the binding equilibrium will be favoured towards the unbound state (see Section 4.4).

The results from this study further support the initial idea of a two-site model as an explanation for the mixed agonist/antagonist effects observed in MCF-7 and CALUX

studies reported in this thesis. The two-site model proposes a primary high affinity site, and a secondary low affinity site. The calculated binding energies from the docking studies reported above support this model, with high affinities being found for ligand binding at the LBC and low ligand binding affinities found for the AF-2 site. The results presented here clearly demonstrate the possibility of dual ER interactions by xenoestrogens; thus, the two-site binding model could be important in explaining and understanding the biological effects of xenoestrogen combinations. This is highlighted by Figure 6.13 which shows E2 bound to the LBC and tetrahydrocurcumin bound to the AF-2 site.

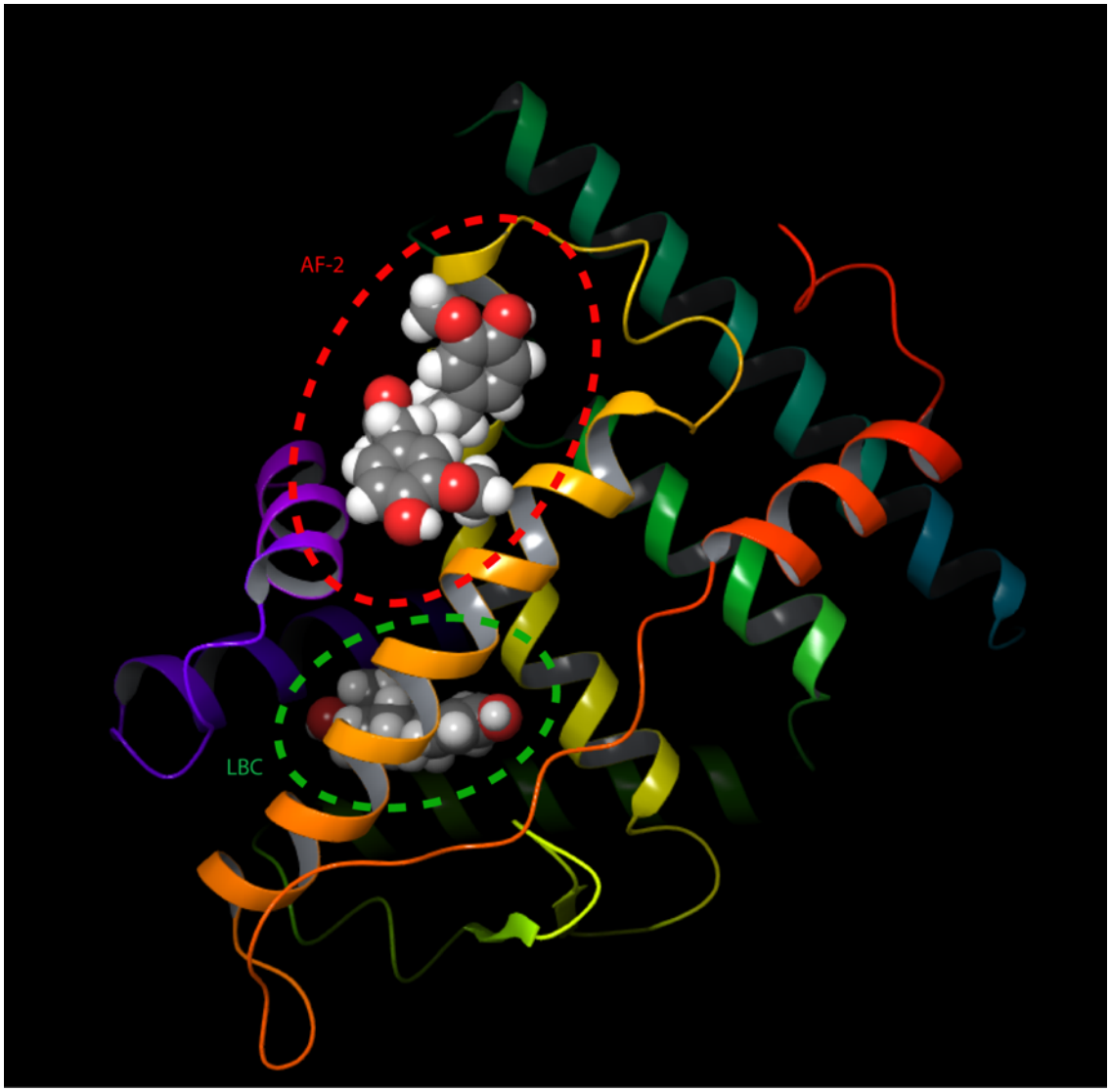


Figure 6.13: Tetrahydrocurcumin bound to the AF-2 (red) site (ERα with E2 complexed at the LBC - green) illustrates the potential for two different ligands to interact with both ER binding sites simultaneously.

6.4.5. Implications for Breast Cancer

Numerous ER structural studies (e.g. x-ray crystallography) have revealed key insights into the function of ER proteins and have provided a platform for the design of pharmaceutical ER- modulators, particularly for the treatment of breast cancer. However, inconsistencies between the biological and structural data highlight that this approach to studying the structure-function relationship does not always paint a complete and accurate picture that fully describes the activity of a ligand in the unique chemical microenvironment of the ER. Indeed, a number of studies, including those presented in Chapter 4, have shown a concentration-dependent mixed agonist/antagonist activity of some xenoestrogens (e.g. genistein) in cultured breast

cancer cells (e.g. MCF-7). *In silico* studies provide a way forward in respect to uncovering the intimate ER/ligand interactions that might help explain this phenomenon.

The *in silico* studies reported in this thesis provide insight into the possible mechanism of mixed agonist/antagonist activity of some xenoestrogens, and supports a departure from the traditional paradigm of xenoestrogen binding to the LBC to a mechanism that involves complex interplay between ER's LBC and the AF-2 site. The AF-2 site is known to play a pivotal role in the activation of ERα and ERβ – mediated gene transcription (Marino *et al*., 2006); thus, the interplay between xenoestrogens and the LBC and AF-2 sites could have important implications for downstream ER-mediated biological activity. For example, xenoestrogen interactions at AF-2 could interfere with the DNA-coactivator-complex formation, which, in turn, may alter ER-mediated gene expression (see Section 4.4). Alternatively, some xenoestrogens could prevent the recruitment of the coactivator protein via their binding to the AF-2 site. Coactivator proteins have enzymatic activity which can facilitate ER degradation via the ubiquitin proteasome degradation pathway; thus, interfering with this process could extend ER occupancy of the target gene promoters which will impact gene transcription (see Section 4.4).

In the context of breast cancer risk, the complex interplay between the dual effects of xenoestrogen interactions with the ER binding sites makes predicting the outcomes of xenoestrogen cocktails almost impossible. For example, the facilitation of AF-2 occupancy, and thus 'spill over' from the LBC site, is likely to be dependent on multiple factors (e.g. xenoestrogen exposures concentration and cocktail composition) which are likely to have high inter-individual variability. Therefore, one person's xenoestrogen cocktail exposure could increase their breast cancer risk (i.e. AF-2 occupancy is not achieved), whilst that same exposure level to one or more particular xenoestrogen cocktail could reduce another person's breast cancer risk (i.e. AF-2 occupancy is achieved). Thus, the total estrogenicity of xenoestrogen cocktails does not necessarily lead to breast cancer development, but could lead to amelioration of the developmental process. This also poses a conundrum when attempting to implement new strategies to reduce breast cancer risk, where the classic approach for regulating chemicals (i.e. by limiting exposure) no longer applies. Indeed, the results reported in this study illustrate the importance of higher xenoestrogen exposure (i.e. a

minimum exposure level) to facilitate AF-2 interactions, depending on the xenoestrogen.

Clearly, understanding the complexity of xenoestrogen cocktails and the interplay between the individual components of these often-complex exposure mixtures at the ER's LBC and AF-2 is important when designing strategies for reducing breast cancer risk. In addition, the present study also highlights the potential for rational drug design approaches involving small E2-mimicking molecules targeted at the functionally important AF-2 site to circumvent competition with the cognate LBC site. This approach could not only improve the treatment of breast cancer but could also be used in preventative strategies to reduce breast cancer risk.

6.5. Concluding Remarks

The results presented in this chapter clearly show the possible AF-2 binding of xenoestrogens, further supporting the two-site binding model presented in chapters 4 and 5. The results highlight the high binding affinity of ligands with the LBC and low binding affinity at the AF-2 supporting the requirements of the two-site binding model and the dose-dependence of the responses (i.e. low and high concentrations elicit agonist and antagonist effects, respectively). However, these experiments were performed *in silico* which begs the question: do they predict the *in vivo* effects? While these studies are computationally simulated they do furnish a logical explanation for the mixed agonist/antagonist effects which is supported by previous work on 4-OHT (Jensen*, et al.*, 2004, Wang*, et al.*, 2006). Therefore, this work could be important in the context of breast cancer risk and furthermore supports the idea that phytoestrogens might be important in breast cancer prevention.

Chapter 7 Human Xenoestrogen Exposure Study

7.1. Introduction

Exposure to xenoestrogens might lead to a number of adverse outcomes, including an increased risk of breast cancer development, as reviewed in Chapter 1. Exposure to xenoestrogens is complex, whereby they do not always simply act in unison with one another (e.g. additivity; see Chapters 4, 5 and 6). Xenoestrogens can bind to ERs, activating ER-mediated signalling pathways; however, some xenoestrogens can modulate these effects, opposing the proliferative actions of other xenoestrogens, depending on the exposure concentration and mixture composition (e.g. mixed agonist/antagonist effects; as discussed in Chapters 4, 5 and 6). Therefore, predicting the outcome of exposures to complex mixtures of xenoestrogens is difficult. To complicate matters further, the same exposure may have different effects on pre-pubertal girls, women of childbearing age, and postmenopausal women, because of differences in circulating E2 levels. The aim of this chapter is to investigate xenoestrogen exposure via daily food intake and lifestyle habits of women and pre-pubertal girls, in order to determine whether the predicted magnitude of exposure is cause for concern in the context of breast cancer risk. Knowledge and understanding of these highly complex exposure cocktails may provide a novel opportunity to develop new preventive strategies for reducing breast cancer risk in New Zealand.

7.1.1. Breast Cancer

Breast cancer is one of the most commonly diagnosed cancers in women in both the developed and developing world. It is estimated that worldwide 1.7 million women were diagnosed with breast cancer in 2016 and 535,000 women died from breast cancer in 2016 (the leading cause of cancer death for women in 2016) (Morange-Serrano, 2018). In New Zealand (estimated female population of 2.3 million in 2013) there were 3020 new registrations (28% of all cancer registrations) and 633 deaths

(15% of all cancer deaths) from breast cancer in 2013 The age-standardised incidence of breast cancer was 98.4 per 100,000 standardised to the WHO World Standard Population (MOH, 2012). Interestingly, survival from breast cancer has improved over time with New Zealand exhibiting some of the highest 5-years net survival rates compared to other developed nations (Allemani*, et al.*, 2018). However, breast cancer outcomes vary significantly across the New Zealand population, with the worst outcomes seen in Māori (Cunningham*, et al.*, 2010), Pasifika and people living in socially deprived areas (Haynes*, et al.*, 2008). This suggests that more should be done to improve breast cancer care and prevention in New Zealand. There are other important factors, particularly social factors such as early diagnosis, which could influence the risk of death; for example, participation rates in BreastScreen Aotearoa (New Zealand's breast screening program) are lower among Māori women (Robson*, et al.*, 2017).

7.1.2. Breast Cancer Initiation, Promotion and Progression

Breast cancer is regarded as a systemic disease that initially presents local manifestations and later may advance, in a multistep process, with various hallmarks including rapid proliferation, resistance to cell death, neoangiogenesis, local invasion, remote metastasis, etc. (Fig. 7.1) (Barrett, 1993, Grizzi*, et al.*, 2006, Liu*, et al.*, 2015, Siddiqui*, et al.*, 2015). Initiation is the first step in breast cancer development. Initiators are often chemicals, also known as mutagens, which cause permanent damage to DNA. These can include exposure to environmental risk factors such as carcinogenic chemicals, radiation or even physical stimuli. On the other hand, initiation can occur from internal metabolic processes (e.g. DNA metabolism, see Section 7.1.3.) (Liu*, et al.*, 2015). Promotion is the second step that occurs in the initiated breast cancer cells. The promoters often refer to the compounds that promote the proliferation of the cell into a large number of daughter cells containing the mutation. Promoters do not directly interact with the DNA like initiators, they often bind to receptors on the cell surface (e.g. ERs) that affect intracellular pathways increasing cell proliferation (see Chapter 4). Whilst promoters do not necessarily cause breast cancer on their own, they do increase the clonal expansion of initiated cells that ultimately leads to malignancy (Klaunig*, et al.*, 2000). The third step, progression, refers to the transformations from a benign tumour through to malignancy. Progression is often associated with karyotype changes coupled with increased growth rate, invasiveness, metastasis and alterations in biochemistry and

morphology due to continuing mutations and genetic instability (Oliveira*, et al.*, 2007).

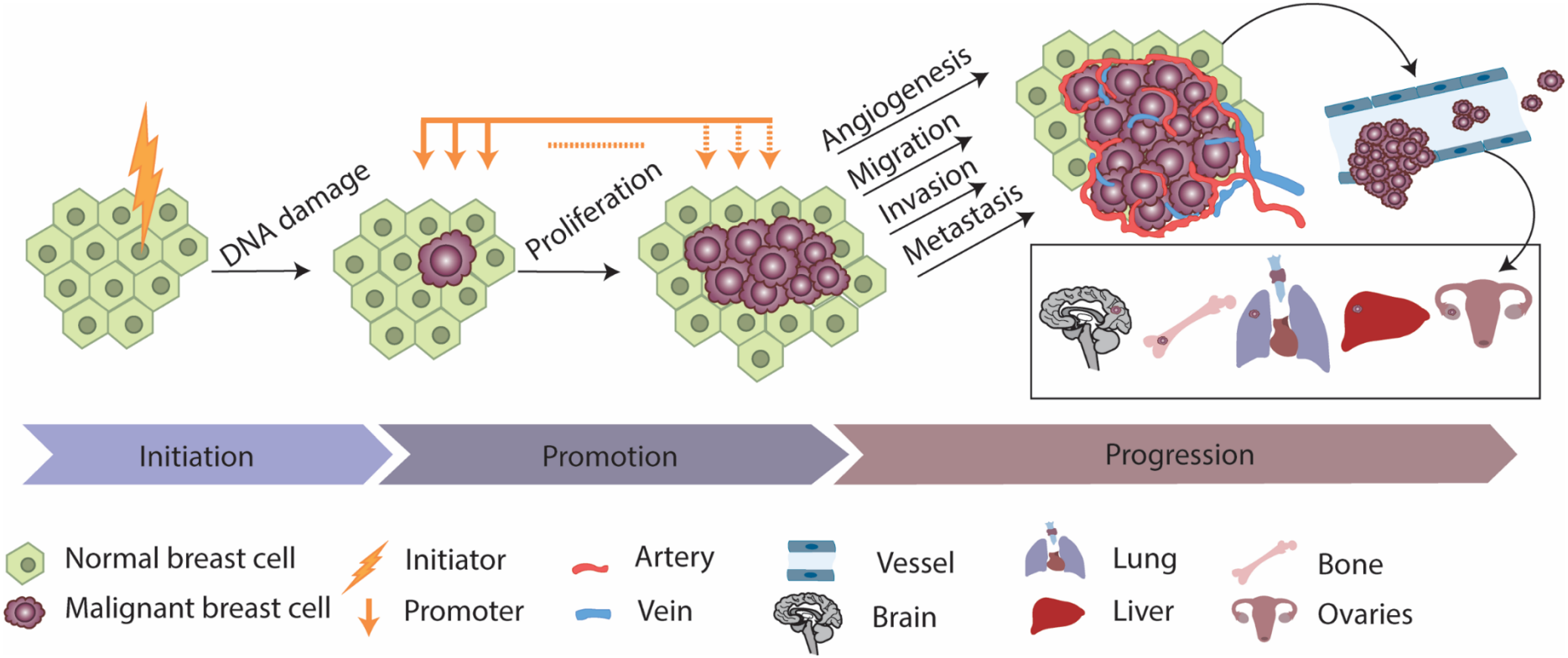


Figure 7.1: Three phases of breast cancer carcinogenesis: initiation upon exposure to a carcinogen, promotion via a proliferative stimuli and progression by an accumulation of increased growth and biochemical alterations (from (Liu, *et al.*, 2015) with permission).

7.1.3. Breast Cancer Risk

Breast cancer risk factors are often thought of as non-modifiable (e.g. genetics, age, etc.); however, modifiable risk factors (e.g. socioeconomic status) are also important (Hiatt*, et al.*, 2018). Whilst this thesis primarily focuses on the adverse effects of xenoestrogen exposures, and the downstream biological implications, the role of the social environment must not be forgotten. For example, a woman's socioeconomic status may influence her food and lifestyle choices, in turn, affecting exposure to xenoestrogens. However, from a biological perspective, breast cancer is thought to develop as a result of accumulated damage induced by both internal (e.g. genetic) and external (e.g. exposure to carcinogens) triggers resulting in initial cell transformation events. The affected cells and tissues then progress through multiple stages, with accompanying alterations in the surrounding tissue likely playing a role in whether the damage leads to breast cancer. These events may occur spontaneously as a by-product of errors in normal processes, such as DNA replication, or potentially though effects of environmental exposures (Hiatt, 2011).

The early procarcinogenic events from endogenous and exogenous processes may be augmented by physiologic conditions such as obesity and other risk factors such as environmental xenoestrogens. It is possible that many such initiative procarcinogenic events may never be entirely preventable because, although potentially modifiable, they are consequences of basic biologic processes (Hiatt, 2011); for example, DNA bases are metabolised to xanthene which is further metabolised by xanthene oxidase to 8-hydroxyguanosine coupled with the release of superoxide ($O_2^{\cdot -}$), a highly reactive species which can result in oxidative DNA damage. Although such biological 'background' DNA damage is unavoidable, highly efficient protective pathways, such as DNA repair and immune surveillance, are effective at reducing the impacts of procarcinogenic events (Bissell*, et al.*, 2011).

Although more needs to be learned about both the mechanisms of breast cancer and the array of risk factors that influence risk; a great deal has already been established (Fig. 7.2). For example, the major factors generally accepted as increasing a woman's risk are older age, inherited genetic mutations (e.g. BRCA1 and BRCA2), and previous breast cancer or benign breast disease. Other risk factors include having a child at an older age (e.g. over 35 years) or never having a child , current/recent use of oral contraceptives or HRT, lack of physical activity (see Section 1.5.2.) and exposure

to ionising radiation (ACS, 2010). While these key breast cancer risks have been identified, for the majority of women diagnosed with breast cancer it is not possible to identify the specific risk factors; however, it is thought that exposure to environmental chemicals may be a key component (IARC, 2008, Lacey*, et al.*, 2009). Interestingly, the observation that the female offspring of immigrants from low-incidence countries within a few generations experience the high incidence rates of countries to which they migrate, illustrates that whichever environmental factors are in play can act to increase rates in genetically similar people in two generations (Hiatt*, et al.*, 2018). Indeed, a US case-control study of Asian women, found that migrants who had lived in the West for a decade or longer had a breast cancer risk 80% higher than more recent migrants (Ziegler*, et al.*, 1993). Therefore, understanding possible environmental and food exposure risk factors could help to mitigate breast cancer risk through implementing food and lifestyle changes. This understanding will help us devise better strategies to help reduce breast cancer risk.

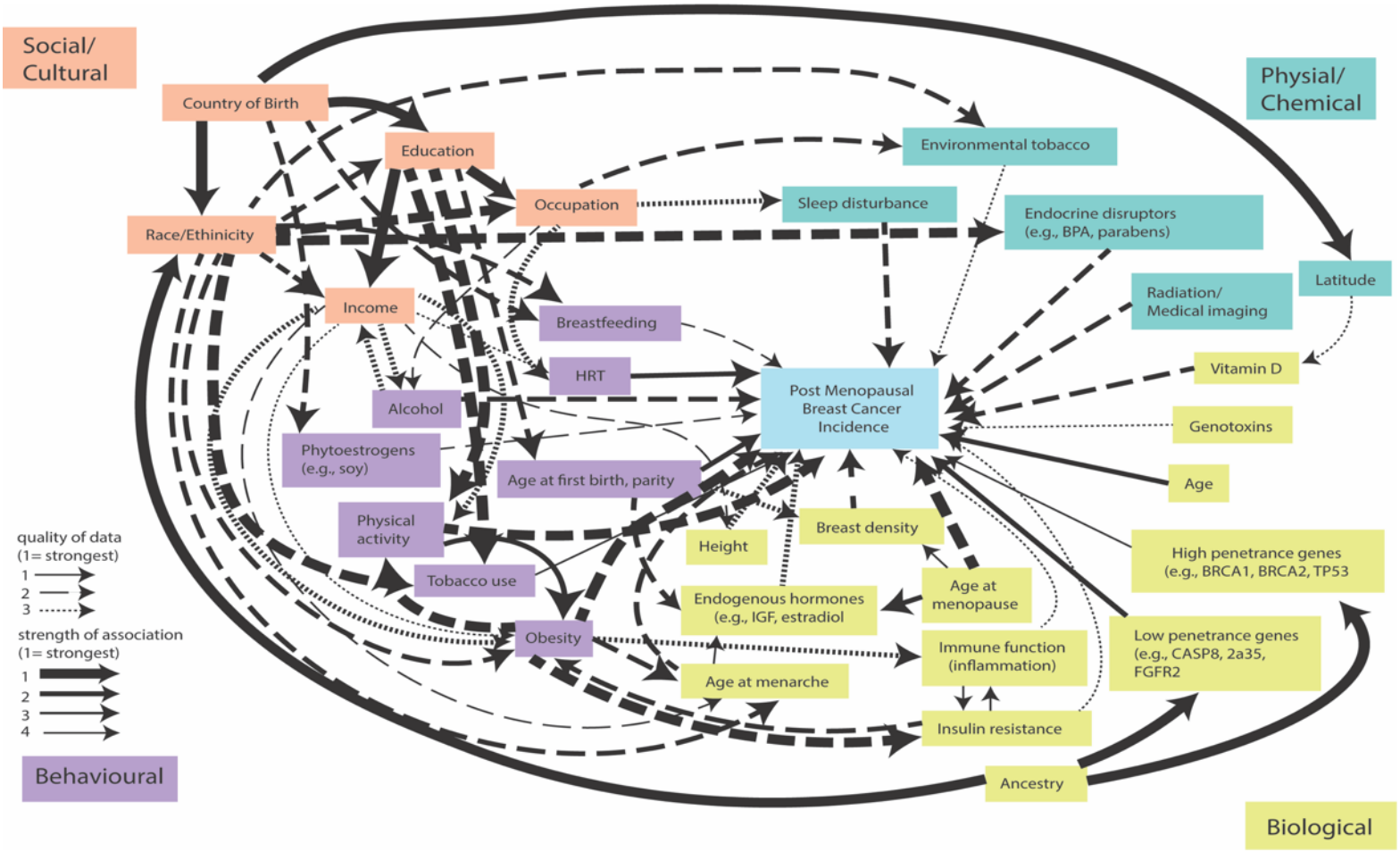


Figure 7.2: Illustration of an evidence-based complex systems model of postmenopausal breast cancer causation. This model displays multiple factors associated with postmenopausal breast cancer causation in four broad domains and shows their interconnections across levels (genes-society) by arrows that indicate strength. This is based on current understanding in the strength of the associations and quality of the data (from (Committee on Breast Cancer and the Environment, 2012) with permission).

7.1.4. Xenoestrogens as Breast Cancer Risk Factors

Estrogen levels in the body are tightly controlled by enzyme feedback mechanisms and gene regulatory processes. This is very important to ensure appropriate control of estrogen-mediated biological activity (Hall*, et al.*, 2001). It is very likely that most xenoestrogens, despite their high structural analogies to E2, may not interact in the same way as E2. This means that xenoestrogens are uncontrolled in terms of their contribution to the total estrogenic load. Xenoestrogens can bind to ERs and imitate endogenous estrogens, exerting estrogenic effects which can interfere with the regulation of growth and proliferation of the cells by endogenous estrogens (Slomczynska, 2008). Again, it must be realised that a single component of a mixture may act via different mechanisms within the ER signalling pathway from another component of the mixture, and these mechanisms can act in unison with or in opposition to one another, thus, predicting the outcome of the exposure to complex xenoestrogen cocktails is difficult (Graham, 2012). One of the most difficult problems relating to xenoestrogens is establishing risk assessment strategies for potential adverse human health effects - this is the case for breast cancer. Current toxicological assessments in most jurisdictions take into consideration the biologic potency of an individual xenoestrogen as well as known exposure scenarios for that xenoestrogen (Slomczynska, 2008); but xenoestrogens are not usually specifically regulated, which means their use, or exposure to them, are not specifically limited. If toxicological testing does pick up estrogenicity (e.g. increased uterine wall thickness in standard animal toxicity testing) it will be considered in the risk assessment. However, the regulatory authorities do not usually consider the possibility of a combined effect of the xenoestrogens; thus, it is possible that the human daily exposure to estrogenic effects of xenoestrogens is a lot higher than anticipated, which could be affecting breast cancer risk.

There are many complicating factors when understanding the combined effects of xenoestrogens: gender and age-related susceptibility to damage (e.g. circulating E2 levels), additional exogenous burden from both natural (e.g. genistein) and synthetic (e.g. BPA) xenoestrogens in food and the environment, and complicated estrogen-mediated biological activity. However, cause-effect relationships between xenoestrogens and breast cancer risk have not been established, except in the case of daughters of women who used DES during pregnancy (Singleton*, et al.*, 2003). It has been shown that DES causes alterations in mammary gland structure and gene

expression in rodents, and was associated with increased breast cancer incidence after the age of 40 in a U.S. cohort of women who were exposed *in utero* (Hoover*, et al.*, 2011).

7.1.5. Importance of Xenoestrogen Regulations

Interestingly, most jurisdictions (e.g. USA, EU) have not limited the use of most xenoestrogens because risk assessments based on exposure to individual compounds (e.g. BPA (EFSA, 2015, WHO, 2010)) have not provided sufficient grounds for regulatory control. Canada, the USA, and many more countries have, however, made an unprecedented move to ban BPA used to manufacture babies' bottles on the grounds that milk formula-fed babies are likely to be a key target group due to their sensitive developmental stage, high BPA intake per body weight, and their sole source of food is likely to be contaminated with BPA (Almeida*, et al.*, 2018, JRC/IHCP, 2010). This will be discussed in greater detail in Section 7.4.6.1.

7.1.6. Human Health Benefits of Xenoestrogens

Just because something is estrogenic does not necessarily mean it will have adverse health effects. Some groups of xenoestrogens, particularly phytoestrogens, might have beneficial effects which could reduce the risk of breast cancer in women, help to alleviate postmenopausal symptoms, and reduce the risk of cardiovascular disease, atherosclerosis and cancer generally (Thomson*, et al.*, 2003). However, because of the persistence of many xenoestrogens in the environment, high potential for human exposure, and the accumulation in biological matrices, interest should be maintained in both determining modes of action and establishing the risk from these chemicals (Singleton*, et al.*, 2003). On the other hand, there is great concern surrounding the link between combinations of xenoestrogens and breast cancer – particularly in adults. Breast cancer incidence currently varies widely around the world, with incidence rates increasing in some countries (e.g. some northern and western European countries) whilst incidence rates in other countries (e.g. France and Norway) are decreasing. These differences in trends are likely a result of different patterns of risk factors (DeSantis*, et al.*, 2015) which could be, in part, linked to exposure to environmental and food xenoestrogens.

7.1.7. Exposure to Xenoestrogens

The most common mode of exposure to xenoestrogens is via the diet; e.g. ingestion of foods containing naturally occurring xenoestrogens (e.g. phytoestrogens) and leeching of monomers from polycarbonate plastics from food packing (e.g. BPA) (Allmyr*, et al.*, 2006, Allmyr*, et al.*, 2008, Dayan, 2007, Muller*, et al.*, 1998, Waring*, et al.*, 2008). In addition, the dermal application of personal care products such as sunscreens and 'wash off' products (e.g. shampoos and soaps), can also result in exposure to estrogenic antimicrobial agents (e.g. parabens) via absorption through the skin (Chedgzoy*, et al.*, 2002, Darbre*, et al.*, 2008, Dayan, 2007, Hayden*, et al.*, 1997, Janjua*, et al.*, 2004). In addition, dietary supplements are another main source of xenoestrogens (Parnell*, et al.*, 2006) that are often overlooked. Dietary supplements are regulated differently around the world. In New Zealand they are deemed foods and are regulated under the guidelines of the Food Act 1981. Under this Act food does not have to be approved for marketing and does not require efficacy or toxicity testing, it simply has to be fit for purpose; thus, dietary supplements are treated in the same manner. However, this has also led to a significant public misunderstanding where dietary supplements can be thought of as a safer alternative to medicines. For example, soy dietary supplements are often marketed as "safe alternatives for hormone replacement treatments for those battling symptoms of menopause". However, these supplements often have much higher concentrations of soy phytoestrogens (e.g. genistein) compared to the concentrations found in whole soy products (e.g. soy beans) (Burke*, et al.*, 2000). The total estrogenic effect of soy dietary supplements is likely higher, thus leading to an effect that could be akin to available pharmaceuticals used to treat menopause. Therefore, women could be unwittingly exposing themselves to high levels of xenoestrogens which could potentially be adding to breast cancer risk.

7.1.8. Understanding the Role of Xenoestrogens in Breast Cancer Prevention

Prevention strategies are crucial if the global breast cancer burden is to be reduced. It has recently been suggested that about one third to a half of diagnosed breast cancers in the Western world could be avoided by practicing healthy lifestyles, such as eating a healthy diet rich in plant-based products (remember genistein is a plant phytoestrogen) (Bouker*, et al.*, 2000, Chang*, et al.*, 2013, Ingram*, et al.*, 1997). Indeed, diets containing plenty of fruits and vegetables have been related to a decreased risk of carcinogenesis, and phytoestrogens are thought to exert

chemopreventive effects (Chang, *et al.*, 2013, Iwasaki, *et al.*, 2008, Magne Nde, *et al.*, 2015). Some cell culture experiments and animal studies have suggested an anti-cancer action of phytoestrogens, while epidemiological studies have found limited, inconsistent and even controversial associations between dietary phytoestrogen consumption and breast cancer risk (Adebamowo, *et al.*, 2005, Chang, *et al.*, 2013, Fink, *et al.*, 2007, Magne Nde, *et al.*, 2015, Touvier, *et al.*, 2013, Travis, *et al.*, 2008, Wang, *et al.*, 2014, Yamamoto, *et al.*, 2003, Zamora-Ros, *et al.*, 2013, Zhu, *et al.*, 2011).

The lower age-standardised incidence rates of breast cancer in some Asian countries, and the increase in breast cancer incidence in Asian women who have migrated to the West having adopted western dietary habits, have suggested a possible protective effect of dietary phytoestrogens. Some investigators have attributed the health benefits for Asian regions to the traditionally high intake of soy foods containing high levels of phytoestrogens (e.g. genistein) (Goodman, *et al.*, 2009, Hedelin, *et al.*, 2008, Lee, *et al.*, 2009, Magne Nde, *et al.*, 2015, Peeters, *et al.*, 2003, Verheus, *et al.*, 2007).

A recent review of dietary intake of phytoestrogens and breast cancer risk found inconsistent associations between the consumption of phytoestrogens and decreased incidence of mammary tumours in Asian populations (i.e. high soy consumption) compared to Western countries (i.e. low soy consumption) (Sak, 2017). Interestingly, this study showed the protective association of phytoestrogens with breast cancer (see Section 1.4.1) might only occur in females who had consumed soy foods during childhood and adolescence. This suggests the consumption of soy phytoestrogens is particularly important during breast development. Thus, understanding these important windows for phytoestrogen exposure could help develop new breast cancer prevention strategies (Sak, 2017, Zhang, *et al.*, 2010).

7.1.9. Biological Regulation of Xeneostrogens

When considering the effects of xenoestrogens one must consider not only exposure to these compounds but also the role of endogenous estrogens. The levels of endogenous E2 vary significantly throughout a woman's life, from 0 – 1.4 x 10^{-10} M at pre-puberty and post menopause (Cummings, *et al.*, 1998, Elmlinger, *et al.*, 2002) to between 9.9 x 10^{-11} – 1.6 x 10^{-9} M (Cummings, *et al.*, 1998) in a woman's child bearing years. Therefore, one cannot fully assess the biological impact of

xenoestrogens without considering circulating E2 levels. For example, xenoestrogens are likely to significantly contribute to a postmenopausal woman's total estrogenic load compared to a woman of child-bearing age based on their circulating endogenous E2 levels (Patisaul*, et al.*, 2010). In addition, xenoestrogens likely circumvent the normal E2-mediated feedback mechanisms, evading biological control. Thus, for postmenopausal women and pre-pubertal girls, only a small proportion of their estrogenicity is, in theory, controlled by these mechanisms and a majority of their estrogenicity can only be 'switched off' via metabolic processes (e.g. glucuronidation) (Liehr*, et al.*, 1998). Therefore, postmenopausal women and girls could be more susceptible to the biological influences of xenoestrogens.

Further adding to the complexity is the individual variability between xenoestrogen uptake, metabolism and excretion. Xenoestrogens are frequently taken up from the gastrointestinal tract, transported to the liver where they are metabolised – often by hydroxylation or glucuronidation – and then normally excreted as inactivate glucuronides in the urine or bile. This could mean that the effects of xenoestrogens are lesser in women who metabolise xenoestrogens rapidly compared with women who are slower metabolisers. Therefore, a 'one size fits all' approach is unlikely to be relevant when devising new risk assessment strategies for xenoestrogen exposures (Thomson, 2005).

7.1.10. Risk Assessment of Xenoestrogen Exposures

If a person is not exposed to a hazard, in this case a xenoestrogen, then they will suffer no adverse effects as a result of that hazard, and there is no risk to them. Risk assessment is a scientifically based process, invaluable for defining the importance of any particular hazard (Thomson, 2005). In terms of xenoestrogen exposure assessments, one must collectively consider exposure (e.g. level, frequency) which determines the dose, and the individual variables (e.g. genetic makeup, age) to determine the risk associated with that particular exposure (Fig. 7.3) (Thomson, 2005). In this Chapter a comprehensive risk assessment combines new and existing data on food concentration and food consumption for each xenoestrogen. Exposure is combined with relative estrogenic potency information, from published bioassay data and data presented in this thesis (Chapter 5), to estimate risk, relative to normal circulating endogenous E2 levels, to determine total estrogenic loads for women and pre-pubertal girls.

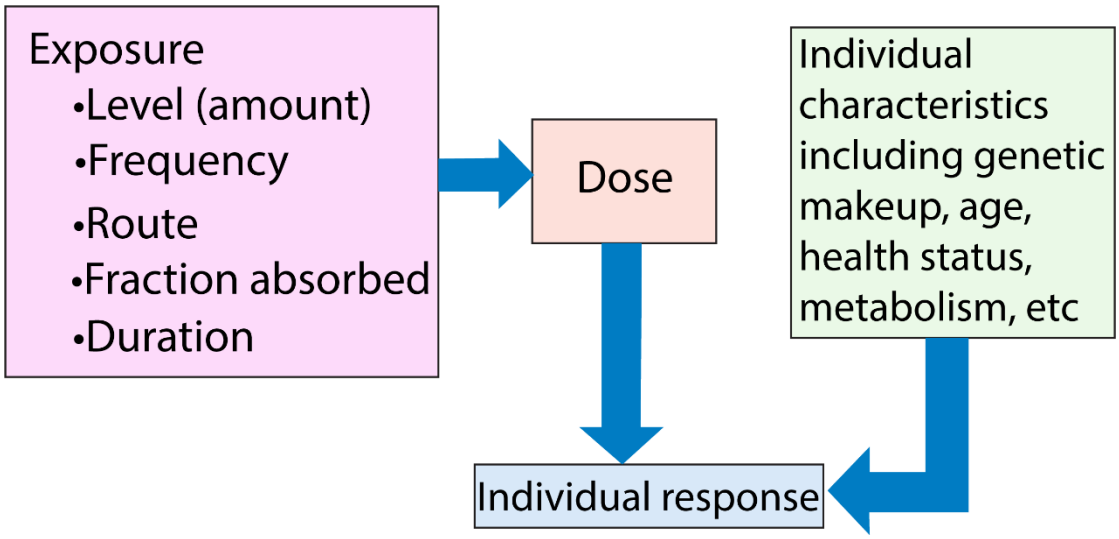


Figure 7.3: Schematic representation of xenoestrogen risk assessment. An individual's response is based on exposure which leads to a specific dose, combined with individual characteristics, gives an estrogenic response.

The aims of this study were (i) to estimate the exposure of a random sample of Canterbury women (and the daughters of women with pre-pubertal daughters) to xenoestrogens, using a daily life habits questionnaire and, (ii) in a smaller non-random sample of women, to compare the findings from the questionnaire with a blood analysis study.

7.2. Experimental (Survey) Approach

A survey of food and lifestyle habits, using a self-administered questionnaire, was carried out to assess xenoestrogen exposure in women and pre-pubertal girls (Group 1). Adult participants (target n=250) were randomly selected from the general and Māori Electoral Rolls and were asked to fill in the questionnaire (see Section 2.2.11.). If applicable the women also completed a questionnaire on behalf of their pre-pubertal daughters. Women were only asked to fill in a 'daughter questionnaire' if their daughter had not had her first period (e.g. menarche). If a woman had more than one pre-pubertal daughter she was asked to fill in the questionnaire for her eldest daughter. In addition, a smaller group of women (Group 2; target n=50) were recruited from UC staff and student email lists to complete the same questionnaire as the other women in conjunction with a blood analysis study, to investigate links between daily diet and lifestyle habits, and circulating xenoestrogens in the blood. As

in Group 1, the women were asked to complete a questionnaire on behalf of their pre-pubertal daughters, if applicable; however, their daughters were not required to provide a blood sample.

7.2.1. Study Population

The study population and sub-populations are described below. Young women were included in the study because of the possibility that risk factors operate much earlier than people had initially thought. The electoral roll only includes people from 18, thus, the first subgroup included 18 and 19-year olds. The age-range for the adult women (18-69 years) was chosen based on available data from the New Zealand Electoral Roll which includes women from age 18 years. The female population begins to decline from about age 69 years; which limited the size of the older (e.g. 69 years and older) sub-population. Therefore, the upper age limit was set at 69 years. The subgroups selected for pre-pubertal girls were based on the available data in the New Zealand Total Diet Survey. Pre-pubertal girls were included to obtain an indication about the similarities between the mother and child's daily habits (i.e. do their daily habits reflect their mothers). Therefore, with a small group of pre-pubertal girls to choose from all pre-pubertal girls were included in the total population calculations; thus, data were also collected for 7-10 years and 4 years subgroups and estimates included data ranges using the parameters from each of the subgroups outlined below.

Exposure assessments were undertaken for the following sub-populations:

- Adult females 18-29 years, 30-39 years, 40-49 years, 50-59 years and 60-69 years.
- Pre-pubertal girls 0-12 months, mean body weight 9 kg
- Pre-pubertal girls 1-3 years, mean body weight 13 kg
- Pre-pubertal girls 5-6 years, mean body weight 23 kg
- Pre-pubertal girls 11-14 years, mean body weight 55 kg

7.2.2. Questionnaire study

The basic design was based on a questionnaire study which determined daily exposure to xenoestrogens. These were then multiplied by the quantified food servings or personal care product application and xenoestrogen concentration data. Blood

volumes were calculated and used to determine circulating xenoestrogen concentrations (in EQ) and endogenous E2 concentrations. The sum of the circulating xenoestrogen concentration and endogenous E2 concentration determined the total daily estrogenic loads (see Section 2.2.11.10.).

Two possible xenoestrogen exposure scenarios were considered, representing the average and worst-case scenario exposure situations. In the average exposure scenario, the total estrogenic load was calculated by adding the calculated average circulating xenoestrogen (in EQ) levels with the calculated circulating endogenous E2 level. In the WCS, the total estrogenic load was calculated by adding the calculated maximum circulating xenoestrogen exposure levels with the calculated circulating endogenous E2 level.

7.2.3. Blood Analysis Study

A 20 mL blood sample was taken from each participant (see Section 2.2.12.1.). The serum from each sample was removed (see Section 2.2.12.2.) and ether-extracted (see Section 2.2.12.3.) to isolate the xenoestrogens. Each sample was tested and analysed for the 13 xenoestrogens using LC-MS (see section 2.2.12.4.). Bruker LC-MS Software was used to observe the collected data and determine whether the xenoestrogens were present in the samples. A positive detection of a xenoestrogen was determined if the mass ion from the blood sample had the same mass ion (within two decimal places) to the standard, in addition to having the same retention time (see Section 2.2.12.4.).

7.3. Results

The questionnaire results from Groups 1 and 2 clearly demonstrate the increase in total estrogenic loads from xenoestrogen exposures. This calculated xenoestrogen exposure has a significant impact on the postmenopausal women and pre-pubertal girl subgroups. Hormone-based medicines clearly contribute significantly to the total estrogenic loads in all subgroups of women, which was expected. In addition, the blood analysis part of the study clearly demonstrates the complex exposures from women's daily food and lifestyle habits.

7.3.1. Questionnaire Response Rates – Group 1

There were 750 participants selected from the electoral roll. Of these, 35 were not currently residing at the address indicated on the electoral roll and 3 were living overseas. Of the remaining 712 women, 227 (32%) returned a completed questionnaire and 34 (15%) of these women also returned a completed questionnaire on behalf of their pre-pubertal daughter. The numbers of responses and percentages of total responses for each subgroup in Group 1 are presented in Tables 7.1 and 7.2

Table 7.1: Responses by age distribution for women participants

Participants (age range)	Number of responses returned	Percentage of total responses
18-29	20	9
30-39	31	14
40-49	51	22
50-59	71	31
60-69	54	24
Total	227	100

Table 7.2: Responses by age distribution for pre-pubertal girls included in the study

Participants (age range)	Number of responses returned	Percentage of total responses
0-12 months	3	9
1-3 years	4	12
4 years	2	6
5-6 years	6	17
7-10 years	12	36
11-14 years	5	14
Age unknown*	2	6
Total	34	100

*Date of birth not given

7.3.2. Exposure to Xenoestrogens – Group 1

7.3.2.1. Women

The results of the calculated xenoestrogen exposures in EQ, circulating E2 levels and total estrogenic loads, also in EQ, are presented in Table 7.3 for each of the subgroups. Each subgroup had similar calculated xenoestrogen exposure ranges;

however, they had different percentage contributions to the daily estrogenic loads. For example, the calculated xenoestrogen exposures made up a higher proportion of the total estrogenic loads for postmenopausal subgroups (e.g. >50 years) compared to subgroups of child-bearing age (e.g. <50 years). Not surprisingly, the calculated WCS xenoestrogen exposures had a much greater contribution to the total estrogenic loads compared with the average xenoestrogen exposure calculations.

Table 7.3: Calculated total daily xenoestrogen exposure in EQ, circulating E2 and total estrogenic loads for women (Group 1) based on average and WCS exposures.

Participant's age range	Calculated xenoestrogen exposure in EQ (M)	Calculated WCS xenoestrogen exposure in EQ (M)	Circulating E2 levels (M)	Calculated total daily estrogenic load in EQ (M)	Calculated total daily WCS estrogenic load in EQ (M)
18-29	$2.0 \times 10^{-11} – 4.0 \times 10^{-11}$	7.3×10^{-10}	$9.9 \times 10^{-11} – 1.6 \times 10^{-9}$	$1.2 \times 10^{-10} – 1.7 \times 10^{-9}$	$8.4 \times 10^{-10} – 2.3 \times 10^{-9}$
30-39	$3.3 \times 10^{-11} – 4.4 \times 10^{-11}$	2.3×10^{-9}	$9.9 \times 10^{-11} – 1.6 \times 10^{-9}$	$1.3 \times 10^{-10} – 1.7 \times 10^{-9}$	$2.5 \times 10^{-9} – 4.0 \times 10^{-9}$
40-49	$3.3 \times 10^{-11} – 4.4 \times 10^{-11}$	2.4×10^{-9}	$9.9 \times 10^{-11} – 1.6 \times 10^{-9}$	$1.3 \times 10^{-10} – 1.7 \times 10^{-9}$	$2.5 \times 10^{-9} – 4.0 \times 10^{-9}$
50-59	$3.4 \times 10^{-11} – 4.4 \times 10^{-11}$	2.4×10^{-9}	$0.0 – 1.5 \times 10^{-10}$	$3.4 \times 10^{-11} – 1.9 \times 10^{-10}$	$2.4 \times 10^{-9} – 2.6 \times 10^{-9}$
60-69	$2.6 \times 10^{-11} – 4.4 \times 10^{-11}$	1.4×10^{-9}	$0.0 – 3.7 \times 10^{-11}$	$2.6 \times 10^{-11} – 8.1 \times 10^{-11}$	1.4×10^{-9}*
All ages	$3.4 \times 10^{-11} – 4.4 \times 10^{-11}$	2.4×10^{-9}	$0.0 – 1.6 \times 10^{-9}$	$3.4 \times 10^{-11} – 1.7 \times 10^{-9}$	$2.4 \times 10^{-9} – 4.0 \times 10^{-9}$

*both values were the same

In addition, daily xenoestrogen exposures were calculated for women who were using hormone-based contraceptives or HRT. The resulting xenoestrogen intakes for the 5 subgroups are shown in Tables 7.4 and 7.5. Interestingly, some postmenopausal women included in the study were taking hormone-based contraceptives as a form of HRT to treat menopausal symptoms; thus, calculations for all subgroups are included. As expected, both hormone-based medications increased the total estrogenic load significantly, with an increase of between 1.4 - 7.1 x 10^4 for the average exposure with hormone-based contraceptives. On the other hand, HRT increased the average daily total estrogenic load 1000 fold. Whilst the calculated WCS xenoestrogen exposures increased the total estrogenic loads, the increases were negligible compared to the increases observed for hormone-based medications. Therefore, the percentage contribution of the WCS calculated xenoestrogen exposures to the total estrogenic loads were almost uniform across subgroups.

Table 7.4: Calculated total daily xenoestrogen exposure in EQ, circulating E2 and total estrogenic loads for women (Group 1) using hormone-based contraceptives based on average and WCS exposures.

Participant's age range	Calculated xenoestrogen exposure in EQ (M)	Calculated WCS xenoestrogen exposure in EQ (M)	Circulating E2 levels (M)	Calculated total daily estrogenic load in EQ (M)	Calculated total daily WCS estrogenic load in EQ (M)
18-29	$1.4 \times 10^{-8} – 2.4 \times 10^{-8}$	$1.5 \times 10^{-8} – 2.5 \times 10^{-8}$	$9.9 \times 10^{-11} – 1.6 \times 10^{-9}$	$1.4 \times 10^{-8} – 2.6 \times 10^{-8}$	$1.5 \times 10^{-8} – 2.7 \times 10^{-8}$
30-39	$1.4 \times 10^{-8} – 2.4 \times 10^{-8}$	$1.6 \times 10^{-8} – 2.6 \times 10^{-8}$	$9.9 \times 10^{-11} – 1.6 \times 10^{-9}$	$1.4 \times 10^{-8} – 2.5 \times 10^{-8}$	$1.6 \times 10^{-8} – 2.8 \times 10^{-8}$
40-49	$1.4 \times 10^{-8} – 2.4 \times 10^{-8}$	$1.6 \times 10^{-8} – 2.7 \times 10^{-8}$	$9.9 \times 10^{-11} – 1.6 \times 10^{-9}$	$1.4 \times 10^{-8} – 2.6 \times 10^{-8}$	$1.6 \times 10^{-8} – 2.8 \times 10^{-8}$
50-59	$1.4 \times 10^{-8} – 2.4 \times 10^{-8}$	$1.6 \times 10^{-8} – 2.6 \times 10^{-8}$	$0.0 – 1.5 \times 10^{-10}$	$1.4 \times 10^{-8} – 2.4 \times 10^{-8}$	$1.6 \times 10^{-8} – 2.7 \times 10^{-8}$
60-69	$1.5 \times 10^{-8} – 2.6 \times 10^{-8}$	$1.6 \times 10^{-8} – 2.7 \times 10^{-8}$	$0.0 – 3.7 \times 10^{-11}$	$1.5 \times 10^{-8} – 2.6 \times 10^{-8}$	$1.6 \times 10^{-8} – 2.7 \times 10^{-8}$
All ages	$1.4 \times 10^{-8} – 2.4 \times 10^{-8}$	$1.6 \times 10^{-8} – 2.7 \times 10^{-8}$	$0.0 – 1.6 \times 10^{-9}$	$1.4 \times 10^{-8} – 2.6 \times 10^{-8}$	$1.6 \times 10^{-8} – 2.8 \times 10^{-8}$

Table 7.5: Calculated total daily xenoestrogen exposure in EQ, circulating E2 and total estrogenic loads for women (Group 1) using HRT based on average and WCS exposures.

Participant's age range	Calculated xenoestrogen exposure in EQ (M)	Calculated WCS xenoestrogen exposure in EQ (M)	Circulating E2 levels (M)	Calculated total daily estrogenic load in EQ (M)	Calculated total daily WCS estrogenic load in EQ (M)
18-29	*	*	$9.9 \times 10^{-11} – 1.6 \times 10^{-9}$	*	*
30-39	*	*	$9.9 \times 10^{-11} – 1.6 \times 10^{-9}$	*	*
40-49	$4.7 \times 10^{-7} – 1.5 \times 10^{-6}$	$4.7 \times 10^{-7} – 1.5 \times 10^{-6}$	$9.9 \times 10^{-11} – 1.6 \times 10^{-9}$	$4.7 \times 10^{-7} – 1.5 \times 10^{-6}$	$4.7 \times 10^{-7} – 1.5 \times 10^{-6}$
50-59	$4.7 \times 10^{-7} – 1.5 \times 10^{-6}$	$4.7 \times 10^{-7} – 1.5 \times 10^{-6}$	$0.0 – 1.5 \times 10^{-10}$	$4.7 \times 10^{-7} – 1.5 \times 10^{-6}$	$4.7 \times 10^{-7} – 1.5 \times 10^{-6}$
60-69	$5.0 \times 10^{-7} – 1.6 \times 10^{-6}$	$5.0 \times 10^{-7} – 1.6 \times 10^{-6}$	$0.0 – 3.7 \times 10^{-11}$	$5.0 \times 10^{-7} – 1.6 \times 10^{-6}$	$5.0 \times 10^{-7} – 1.6 \times 10^{-6}$
All ages	$4.7 \times 10^{-7} – 1.5 \times 10^{-6}$	$4.7 \times 10^{-7} – 1.5 \times 10^{-6}$	$0.0 – 1.6 \times 10^{-9}$	$4.7 \times 10^{-7} – 1.5 \times 10^{-6}$	$4.7 \times 10^{-7} – 1.5 \times 10^{-6}$

* women not receiving HRT

7.3.2.2. Pre-pubertal Girls

The results of the calculated xenoestrogen exposures in EQ, circulating E2 levels and total estrogenic loads, also in EQ, for the pre-pubertal girls are presented in Table 7.6. As for the women, average and WCS xenoestrogen exposures were calculated for each subgroup of pre-pubertal girls. The ranges of calculated xenoestrogen exposures were more variable than those for the women in the study. Girls in the 11-14 year subgroup were exposed to a similar range of xenoestrogen concentrations as the women. The calculated xenoestrogen exposures for the 1-3 year and 5-6 year subgroups were found to have the highest percentage contribution to the total estrogenic load. The 0-12 month subgroup was also exposed to similar concentrations of xenoestrogens. The calculated WCS xenoestrogen exposures significantly increased the total estrogenic loads of all subgroups.

Table 7.6: Calculated total daily xenoestrogen exposure, circulating E2 and total estrogenic loads for pre-pubertal girls (Group 1) based on average and WCS.

Participant's age range	Calculated xenoestrogen exposure in EQ (M)	Calculated WCS xenoestrogen exposure in EQ (M)	Circulating E2 levels (M)	Calculated total daily estrogenic load in EQ (M)	Calculated total daily WCS estrogenic load in EQ (M)
0-12 months	$2.3 \times 10^{-11} – 1.5 \times 10^{-10}$	7.9×10^{-9}	$0.0 – 5.1 \times 10^{-11}$	$2.3 \times 10^{-11} – 2.3 \times 10^{-10}$	$7.9 \times 10^{-9} – 8.0 \times 10^{-9}$
1-3 years	$1.2 \times 10^{-10} – 2.0 \times 10^{-10}$	4.2×10^{-9}	$0.0 – 7.3 \times 10^{-11}$	$1.2 \times 10^{-10} – 2.7x 10^{-10}$	4.2×10^{-9}- 4.3×10^{-9}
5-6 years	$8.4 \times 10^{-11} – 1.5 \times 10^{-10}$	1.3×10^{-9}	$0.0 – 7.3 \times 10^{-11}$	$8.4 \times 10^{-11} – 2.3 \times 10^{-10}$	$1.3 \times 10^{-9} – 1.4 \times 10^{-9}$
11-14 years	$2.3 \times 10^{-11} – 5.1 \times 10^{-11}$	5.1×10^{-10}	$0.0 – 1.3 \times 10^{-9}$	$2.3 \times 10^{-11} – 1.3 \times 10^{-9}$	$5.1 \times 10^{-10} – 1.8 \times 10^{-9}$
All ages	$3.4 \times 10^{-11} – 2.5 \times 10^{-10}$	$1.7 \times 10^{-9} – 8.4 \times 10^{-9}$	$0.0 – 1.3 \times 10^{-9}$	$3.4 \times 10^{-11} – 1.5 \times 10^{-9}$	$1.7 \times 10^{-9} – 9.7 \times 10^{-9}$

7.3.3. Hormone-based Medications and Dietary Supplement Use – Group 1

The questionnaire included questions about contraceptives and HRT and dietary supplements, as these are some of the main sources of xenoestrogens to which women are exposed on a daily basis. Of the 227 participants 32 were using hormone-based contraceptives; a majority of these women were under the age of 40 years (Table 7.7). In addition, 11 participants were taking a form of HRT, with 100% these women over the age of 40 years (Table 7.8). Interestingly, 85 participants (37%) were taking dietary supplements, with those over age 40 years being the greatest consumers (78%) (Table 7.9).

Table 7.7: Participants from Group 1 who had used hormone contraceptives.

Participant's age range	Number of positive responses returned	Percentage of total responses
18-29	11	34
30-39	9	28
40-49	8	25
50-59	4	13
60-69	0	0
Total	32	14

Table 7.8: Participants from Group 1 who had used HRT.

Participant's age range	Number of positive responses returned	Percentage of total responses
18-29	0	0
30-39	0	0
40-49	2	18
50-59	5	46
60-69	4	36
Total	11	5

Table 7.9: Participants from Group 1 who had consumed dietary supplements.

Participant's age range	Number of positive responses returned	Percentage of total responses (%)
18-29	11	13
30-39	8	9
40-49	21	25
50-59	23	27
60-69	22	26
Total	85	37

Of the 36 pre-pubertal girls included in this study, 11 (30.6%) were taking dietary supplements. All of the girls taking the supplements were over the age of 5 years (Table 7.10).

Table 7.10: Participants from Group 1 whose daughter was consuming dietary supplements.

Participant's age range	Number of positive responses returned	Percentage of total responses
0-12 months	0	0
1-3 years	0	0
5-6 years	3	50
11-14 years	1	20
Total	11+	30.6

+ Total calculations include pre-pubertal girls who were 4 years old and those from the 7-10 year age group.

7.3.4. Relationship Between Breast Cancer and Exposure to Xenoestrogens in Group 1

To attempt to understand whether some of the participants' food and lifestyle habits were influenced by a previous or current breast cancer diagnosis, participants were asked if they had breast cancer or had previously undergone treatment for breast cancer. Out of 227 participants, 14 women (6%) had either been diagnosed with breast cancer or were currently undergoing treatment for breast cancer at the time of the questionnaire study. Two of these women (14 %) were under the age of 40 years while 86% were over the age of 50 years (Table 7.11).

Table 7.11: Participants from Group 1 who had a history of breast cancer.

Participant's age range	Number of positive responses returned	Percentage of total responses
18-29	1	7
30-39	1	7
40-49	0	0
50-59	6	43
60-69	6	43
Total	14	6

In addition, participants were asked whether they had previously thought about the possible link between xenoestrogens and breast cancer. Of the 227 participants 73 (32 %) had thought about xenoestrogens as possible breast cancer risk factors, while 68 (30 %) had taken measures to eliminate xenoestrogens from their daily food and lifestyle habits (Table 7.12). This suggests that five of the participants who had thought about xenoestrogens as breast cancer risk factors did not change their food and lifestyle habits to reduce breast cancer risk. However, when this was examined in the five subgroups, it is clear this was not always the case. For example, in the 18-29 year subgroup, 7 participants had thought about xeneostrogens as breast cancer risk factors, but 8 participants had taken measures to eliminate them from their daily habits. This suggests that while information is available encouraging women to eliminate xenoestrogens from their diet and lifestyle, people are not always aware of why xenoestrogen exposure might need to be reduced.

Table 7.12: Participants from Group 1 who had thought about xenoestrogens as breast cancer risk factors and had taken measures to eliminate them from their daily habits prior to receiving the questionnaire.

	Number of women who had considered:	
Participant's age range	Breast cancer risk factors	Elimination
18-29	7	8
30-39	11	8
40-49	18	19
50-59	19	18
60-69	18	15
Total	73	68

7.3.5. Relationship Between Breast Cancer Risk and Exposure to Xenoestrogens for Pre-pubertal Girls

Interestingly, 33.3% of the women who completed the questionnaire on behalf of their pre-pubertal daughters had considered xenoestrogens as possible breast cancer risk factors for their daughters. However, 36.1% of women had taken measures to eliminate xenoestrogens from their daughter's daily food and lifestyle habits (Table 7.13).

Table 7.13: Participants from Group 1 who had thought about xenoestrogens as breast cancer risk factors for their daughter and had taken measures to eliminate them from her daily habits prior to receiving the questionnaire.

	Number of women who had considered:	
Participant's age range	Breast cancer risk factors	Elimination
0-12 months	2	2
1-3 years	1	1
5-6 years	1	1
11-14 years	3	3
Total	12^{+}	13

+Total calculations include pre-pubertal girls who were 4 years old and those from the 7-10 year age group.

7.3.6. Exposure to Xenoestrogens – Group 2

7.3.6.1. Women

The results of the calculated xenoestrogen exposures in EQ, circulating E2 levels and total estrogenic loads, also in EQ, are presented in Table 7.14 for each of the subgroups. Interestingly, despite the different sampling strategies, the findings from the questionnaires suggest that both groups of women have similar xenoestrogen exposures. The calculated xenoestrogen exposures made up a higher proportion of the total estrogenic loads for postmenopausal subgroups (e.g. >50 years) compared to women of child-bearing age (e.g. <50 years). Not surprisingly, the calculated WCS xenoestrogen exposures were found to have a much greater contribution to the total estrogenic loads compared the average xenoestrogen exposure calculations.

In addition, the calculated daily xenoestrogen exposures for women using hormone-based contraceptives in Group 2 is presented in Table 7.15. As expected, the hormone-based medications increased the total estrogenic load significantly, with an increase between $1.5 \times 10^3 – 6.6 \times 10^4$ for the calculated average exposure and 1.3×10^3-6.7×10^7 for the calculated WCS exposure. The calculated WCS xenoestrogen exposures increased the total estrogenic loads; however, those increases appear to be negligible compared to the increases observed by hormone-based medications. Therefore, the percentage contribution of the WCS calculated xenoestrogen exposures to the total estrogenic loads were almost uniform across subgroups.

Table 7.14: Calculated total daily xenoestrogen exposure in EQ, circulating E2 and total estrogenic loads for women (Group 2) based on average and WCS exposures.

Participant's age range	Calculated xenoestrogen exposure in EQ (M)	Calculated WCS xenoestrogen exposure in EQ (M)	Circulating E2 levels (M)	Calculated total daily estrogenic load in EQ (M)	Calculated total daily WCS estrogenic load in EQ (M)
18-29	4.0×10^{-11} - 1.1×10^{-9}	2.9×10^{-9}	9.9×10^{-11} – 1.6×10^{-9}	1.4×10^{-10} – 3.2×10^{-9}	2.7×10^{-9} – 4.0×10^{-9}
30-39	4.4×10^{-11} - 2.5×10^{-11}	1.2×10^{-9}	9.9×10^{-11} – 1.6×10^{-9}	1.4×10^{-10} – 1.8×10^{-9}	1.3×10^{-9} – 2.8×10^{-9}
40-49	1.6×10^{-11} - 1.8×10^{-11}	7.3×10^{-10}	9.9×10^{-11} – 1.6×10^{-9}	1.1×10^{-10} – 1.6×10^{-9}	8.4×10^{-10} – 2.3×10^{-9}
50-59	$2.1 \times 10^{-}11$ - 3×10^{-11}	1.5×10^{-10}	0.0 – 1.5×10^{-10}	2.1×10^{-11} – 1.8×10^{-10}	1.5×10^{-10} – 3×10^{-10}
60-69	1.9×10^{-11} – 3×10^{-11}	2.5×10^{-10}	0.0 – 3.7×10^{-11}	1.9×10^{-11} – 6.6×10^{-11}	2.5×10^{-10} – 2.8×10^{-10}
All ages	3.1×10^{-11} – 3.6×10^{-11}	2.4×10^{-9}	0.0 – 1.6×10^{-9}	3.1×10^{-11} – 1.7×10^{-9}	1.1×10^{-9} – 2.8×10^{-9}

Table 7.15: Calculated total daily xenoestrogen exposure in EQ, circulating E2 and total estrogenic loads for women (Group 2) using hormone-based contraceptives based on average and WCS exposures.

Participant's age range	Calculated xenoestrogen exposure in EQ (M)	Calculated WCS xenoestrogen exposure in EQ (M)	Circulating E2 levels (M)	Calculated total daily estrogenic load in EQ (M)	Calculated total daily WCS estrogenic load in EQ (M)
18-29	1.5×10^{-8} – 2.7×10^{-8}	1.8×10^{-8} – 2.9×10^{-8}	9.9×10^{-11} – 1.6×10^{-9}	1.5×10^{-8} – 2.9×10^{-8}	1.8×10^{-8} – 3.0×10^{-8}
30-39	1.5×10^{-8} – 2.6×10^{-8}	1.6×10^{-8} – 2.7×10^{-8}	9.9×10^{-11} – 1.6×10^{-9}	1.5×10^{-8} – 2.8×10^{-8}	1.6×10^{-8} – 2.9×10^{-8}
40-49	1.3×10^{-8} – 2.3×10^{-8}	1.4×10^{-8} – 2.4×10^{-8}	9.9×10^{-11} – 1.6×10^{-9}	1.3×10^{-8} – 2.5×10^{-8}	1.4×10^{-8} – 2.6×10^{-8}
50-59	1.3×10^{-8} – 2.4×10^{-8}	1.4×10^{-8} – 2.4×10^{-8}	0.0 – 1.5×10^{-10}	1.3×10^{-8} – 2.4×10^{-8}	1.4×10^{-8} – 2.4×10^{-8}
60-69	1.4×10^{-8} – 2.4×10^{-8}	1.4×10^{-8} – 2.4×10^{-8}	0.0 – 3.7×10^{-11}	1.4×10^{-8} – 2.4×10^{-8}	1.4×10^{-8} – 2.4×10^{-8}
All ages	1.4×10^{-8} – 2.5×10^{-8}	1.5×10^{-8} – 2.6×10^{-8}	0.0 – 1.6×10^{-9}	1.4×10^{-8} – 2.6×10^{-8}	1.5×10^{-8} – 2.7×10^{-8}

7.3.6.2. Pre-pubertal Girls

The results of the calculated xenoestrogen exposures in EQ, circulating E2 levels and total estrogenic loads, also in EQ, for the 8 pre-pubertal girls in Group 2 are presented in Table 7.16. Again, average and WCS xenoestrogen exposures were calculated for each subgroup of pre-pubertal girls. The ranges of calculated xenoestrogen exposures were more variable compared to the women in the group. The 11-14 year subgroup were exposed to a similar concentration range of xenoestrogens to the women. Interestingly, the calculated xenoestrogen exposures for the 1-3 year and 5-6 year subgroups were found to have the highest percentage contribution to the total estrogenic load. Of the 47 questionnaires returned, no participants had a daughter aged under 12 months. The calculated WCS xenoestrogen exposures significantly increased the total estrogenic loads of all subgroups.

Table 7.16: Calculated total daily xenoestrogen exposure in EQ, circulating E2 and total estrogenic loads for pre-pubertal girls (Group 2) based on average and WCS exposures.

Participant's age range	Calculated xenoestrogen exposure in EQ (M)	Calculated WCS xenoestrogen exposure in EQ (M)	Circulating E2 levels (M)	Calculated total daily estrogenic load in EQ (M)	Calculated total daily WCS estrogenic load in EQ (M)
1-3 years	$1.2 \times 10^{-10} – 4.0 \times 10^{-9}$	4.0×10^{-9}	$0.0 – 5.1 \times 10^{-11}$	$1.2 \times 10^{-10} – 4.1 \times 10^{-9}$	4.0×10^{-9}
5-6 years	$7.0 \times 10^{-11} – 2.4 \times 10^{-10}$	2.4×10^{-10}	$0.0 – 7.3 \times 10^{-11}$	$7.0 \times 10^{-11} – 3.1 \times 10^{-10}$	2.4×10^{-10}
11-14 years	$4.8 \times 10^{-12} – 7.3 \times 10^{-11}$	1.4×10^{-9}	$0.0 – 7.3 \times 10^{-11}$	$4.8 \times 10^{-12} – 1.4 \times 10^{-9}$	$1.4 \times 10^{-9} – 2.7 \times 10^{-9}$
All ages	$3.3 \times 10^{-11} – 4.8 \times 10^{-10}$	6.4×10^{-9}	$0.0 – 1.3 \times 10^{-9}$	$3.3 \times 10^{-11} – 1.8 \times 10^{-9}$	$6.4 \times 10^{-9} – 2.7 \times 10^{-9}$

7.3.7. Hormone-based Medications and Dietary Supplement Use – Group 2

Of the 47 participants 12 (26%) were using hormone-based contraceptives, with a majority of participants under age 40 (Table 7.17). On the other hand, no participants were using a form of HRT. Ten women (21%) had taken a dietary supplement, with an even distribution among subgroups (Table 7.18). Only one (11.1%) pre-pubertal girl had taken a dietary supplement (Table 7.19).

Table 7.17: Participants from Group 2 who had used hormone contraceptives.

Participant's age range	Number of positive responses returned	Percentage of total responses
18-29	3	30
30-39	3	33
40-49	4	44
50-59	2	20
60-69	0	0
Total	12	26

Table 7.18: Participants from Group 2 who had consumed dietary supplements.

Participant's age range	Number of positive responses returned	Percentage of total responses
18-29	3	30
30-39	3	33
40-49	2	22
50-59	2	20
60-69	0	0
Total	10	21

Table 7.19: Participants from Group 2 whose daughter had consumed dietary supplements.

Participant's age range	Number of positive responses returned	Percentage of total responses
0-12 months	*	0
1-3 years	0	0
5-6 years	0	0
11-14 years	0	0
Total	1+	11.1

*indicates no participants in this subgroup; + Total calculation includes pre-pubertal girls who were 4 years old and those from the 7-10 year age group.

7.3.8. Relationship Between Breast Cancer and Exposure to Xenoestrogens in Group 2

To attempt to understand whether some of the participants in Group 2's food and lifestyle habits were influenced by a previous or current breast cancer diagnoses, participants were asked if they had breast cancer or had previously undergone treatment for breast cancer. Of the 47 participants, one woman (2%), who was in the 60-69 year age-group, had been diagnosed with breast cancer at the time of the questionnaire study.

In addition, participants were asked if they had previously thought about the link between xenoestrogens and breast cancer. Out of the 47 participants 21 (45%) had thought about xenoestrogens as possible breast cancer risk factors, while 17 (36%) had taken measures to eliminate xenoestrogens from their daily food and lifestyle habits (Table 7.20). This suggests that four participants who had thought about xenoestrogens as possible breast cancer risk factors did not change their food and lifestyle habits.

Table 7.20: Participants from Group 2 who had thought about xenoestrogens as breast cancer risk factors and had eliminated xenoestrogens from their daily habits prior to receiving the questionnaire.

	Number of women who had considered:	
Participant's age range	Breast cancer risk factors	Elimination
18-29	3	2
30-39	6	5
40-49	3	3
50-59	4	4
60-69	5	3
Total	21	17

7.3.9. Relationship Between Breast Cancer Risk and Exposure to Xenoestrogens for Pre-pubertal Girls

Interestingly, 55.6% of participants who completed a questionnaire for their pre-pubertal daughters had considered xenoestrogens as breast cancer risk factors for their daughters. In addition, 100% of these participants had taken measures to eliminate xenoestrogens from their daughters' daily food and lifestyle habits (Table 7.21). For a majority of participants this included the removal of BPA-containing plastic containers and packaging.

Table 7.21: Participants from Group 2 who had thought about xenoestrogens as breast cancer risk factors for their daughter prior to receiving the questionnaire.

	Number of women who had considered:	
Participant's age range	Breast cancer risk factors	Elimination
0-12 months	*	*
1-3 years	1	1
5-6 years	1	1
11-14 years	2	2
Total	5^{+}	5

*indicates no participants in this subgroup; + Total calculation includes pre-pubertal girls who were 4 years old and those from the 7-10 year age group.

7.3.10. Xenoestrogen Blood Analysis Results

The forty-seven women selected (see Section 2.2.11.) from the UC staff and student lists participated in this part of the study. A 20 mL blood sample was taken from each participant and analysed for the 13 xenoestrogens selected for the study by LC-MS (see Section 2.2.12.). The results of the blood analyses are shown in Tables 7.21 to 7.25. Interestingly, 10 out of the 13 xenoestrogens tested were detected in all 47 blood samples. The method used to select the women for this part of the study was not statistically based as this was a small pilot study, therefore, these results are not statistically based; however, it is interesting that a small sample of women all showed varying levels of different xenoestrogens in their blood. This suggest widespread exposure.

7.3.10.1. Parabens

Methylparaben and butylparaben were detected in 100% and 87.2%, respectively, of blood samples tested (Table 7.22; Fig. 7.4). It was not surprising to find both parabens in the samples given the extensive use of methyl and butylparaben in cosmetics and personal care products (see Section 1.4.3.). On the other hand, benzylparaben (Fig. 7.4) was only detected in one of the samples (2.1%). This suggests that benzylparaben is not as commonly used as an anti-microbial agent in cosmetics and personal care products.

Table 7.22: Blood samples with parabens detected.

	Methylparaben (%)	Butylparaben (%)	Benzylparaben (%)
18-29	100.0	90.0	0.0
30-39	100.0	77.8	0.0
40-49	100.0	88.9	10.0
50-59	100.0	90.0	0.0
60-69	100.0	88.9	0.0
All	100.0	87.2	2.1

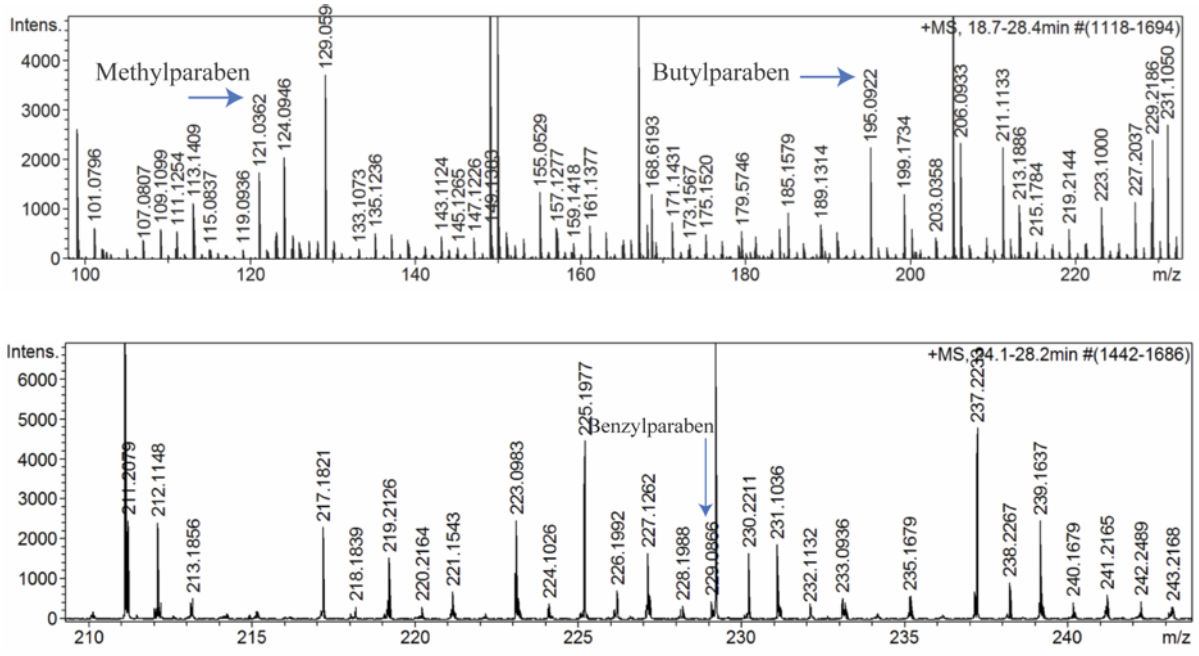


Figure 7.4: Example chromatogram of a blood sample showing the mass ion peaks for methylparaben, butylparaben (top) and benzylparaben (bottom).

7.3.10.2. Endogenous Estrogens

E2 (68%) was the only estrogen detected in any of the blood samples analysed (Table 7.23; Fig. 7.5). Surprisingly, E2 was detected in only 40% of blood samples from the 18-29 year subgroup. Comparatively, E2 was detected in 77.8% - 80.0% of blood samples from the remaining subgroups of women. However, one would expect to find E2 in all blood samples of women up to the age to 50 years based on their theoretical circulating E2 levels and the sufficient detection level of the analytical methodology. There was a change made during the blood collection in that it was found more convenient to centrifuge directly in the vacutainer rather than to transfer it to a centrifuge tube and this could have accounted for that difference. This might be due to a more sufficient serum separation. Estrone and estriol were not detected in any of the 47 blood samples.

Table 7.23: Blood samples with endogenous estrogens detected.

	E2 (%)	Estrone (%)	Estriol (%)
18-29	40.0	0.0	0.0
30-39	77.8	0.0	0.0
40-49	77.8	0.0	0.0
50-59	80.0	0.0	0.0
60-69	77.8	0.0	0.0
All	68.0	0.0	0.0

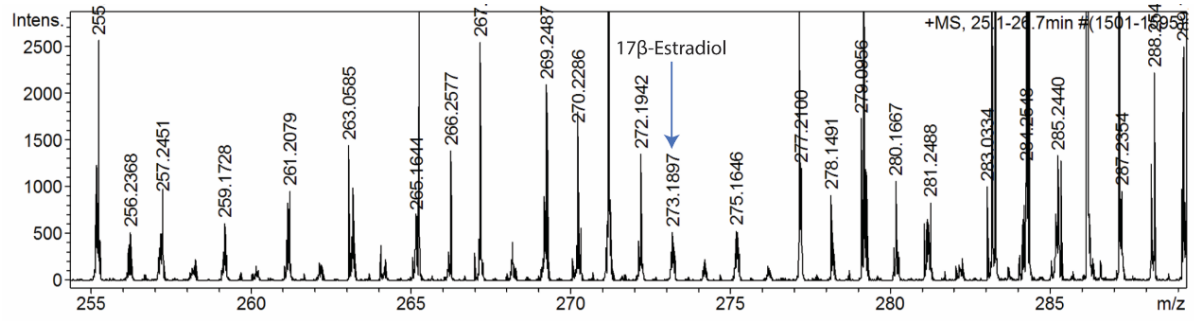


Figure 7.5: Example chromatogram of a blood sample showing the mass ion peak for E2.

7.3.10.3. Synthetic Xenoestrogens

EE2 was detected in one of the 47 blood samples (2.1%; Table 7.24; Fig. 7.6). When compared with the relative questionnaire for that participant, she was taking an EE2 containing oral contraceptive; therefore, it was not surprising to find EE2 in her serum. On the other hand, BPA was not detected in any of the blood samples. This is not surprising as BPA is rapidly metabolised and quickly excreted from the body after exposure, this will be discussed later in Section 7.4.6.1.

Table 7.24: Blood samples with synthetic xenoestrogens detected.

	EE2 (%)	BPA (%)
18-29	10.0	0.0
30-39	0.0	0.0
40-49	0.0	0.0
50-59	0.0	0.0
60-69	0.0	0.0
All	2.1	0.0

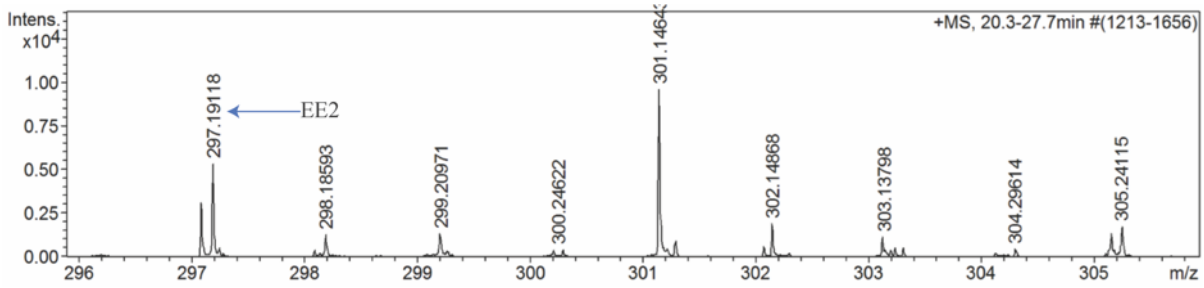


Figure 7.6: Example chromatogram of a blood sample showing the mass ion peak for EE2

7.3.10.4. Phytoestrogens

Phytoestrogens were found in all of the 47 blood samples (Table 7.25). Kaempferol was the most prevalent of the phytoestrogens, with it being detected in 89.4% of blood samples. Interestingly, the mass ion peak for kaempferol often had a higher intensity (Fig. 7.7) compared to the other phytoestrogens (e.g. genistein or daidzein), suggesting that kaempferol is present at higher concentrations in blood. Genistein and daidzein were detected in 34.0% and 29.8% of samples, respectively. Curcumin was detected in 27.7% of samples and the metabolite tetrahydrocurcumin was detected in one sample (2.1%). Interestingly, the sample containing tetrahydrocurcumin (Fig. 7.7) had a much higher peak of curcumin than was observed in the other samples. This suggests that the detection limits were possibly not low enough to detect tetrahydrocurcumin in the other samples.

Table 7.25: Blood samples with phytoestrogens detected.

	Daidzein (%)	Genistein (%)	Kaempferol (%)	Curcumin (%)	Tetrahydrocurcumin (%)
18-29	40.0	20.0	70.0	0.0	0.0
30-39	22.2	66.7	100.0	44.4	0.0
40-49	33.3	33.3	100.0	33.3	0.0
50-59	20.0	22.2	90.0	30.0	11.1
60-69	33.3	34.0	88.9	30.0	0.0
All	29.8	34.0	89.4	27.7	2.1

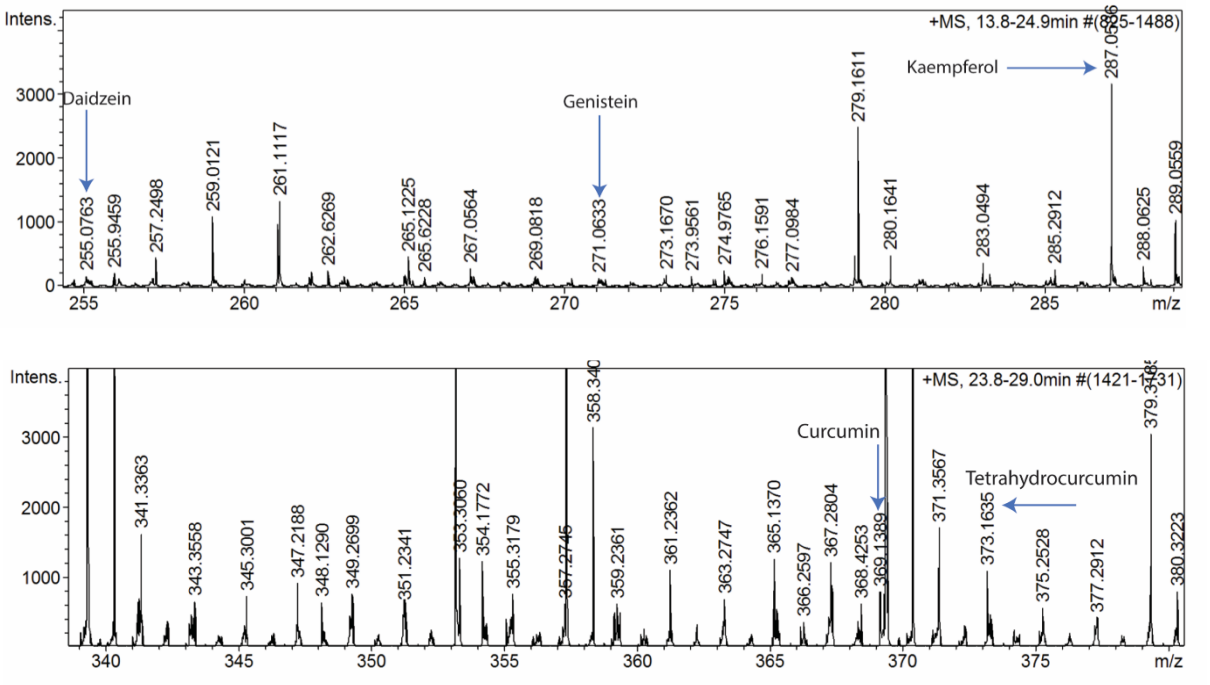


Figure 7.7: Example chromatograms showing mass ion peaks for daidzein, genistein, kaempferol (top), curcumin and tetrahydrocurcumin (bottom).

7.4. Discussion

This study was effectively composed of three sub-studies. The first was a population-based study with randomly selected women (questionnaire study). The second was a group of women selected off UC staff and student lists (blood analysis study). The third was pre-pubertal girls of the women included in groups 1 and 2. They were included because of the possibility that risk factors operate much earlier in life than people had initially thought; thus, exposure to xenoestrogens pre-puberty could translate into an increased breast cancer incidence postmenopausally. In Group 1, postmenopausal women (who have lower levels of endogenous E2) were potentially the most susceptible if exposed to xenoestrogens because of the low E2: xenoestrogen ratio. The average contribution of the calculated xenoestrogen exposures to their total estrogenic loads was considerably higher (65.4%) in these subgroups compared with women of child-bearing age (16.9%). This study also demonstrates the complexity of xenoestrogen exposures from daily food and lifestyle habits, including hormone-based medications and the use of dietary supplements. Indeed, the blood analysis part of the study demonstrated that the xenoestrogens from the women's food and lifestyle were clearly high enough to be present in the blood samples. However, upon comparison between the questionnaire and blood analysis results from this group of women, there was no obvious correlations between a woman's daily habits and the xenoestrogens detected in her blood. For example, genistein and daidzein were not always detected in the blood samples from women who consumed bread on a daily basis. However, this was nott a fully validated study but rather a preliminary investigation and the limits of determination are not known for these studies; therefore, it might be that compounds were present at low concentrations that were not detected by the analytical methodology. This is a study that requires significant modifications. In addition, detection also depended on a number of variables, including the time of presence of a compound in the circulatory system is dependent on the dose and the pharmacokinetics of the compounds. The biological half-lives of xenoestrogens vary significantly (e.g. BPA ~2 h, genistein ~8 h; Table 7.26); therefore, a compound that has a shorter half-life is going to disappear very quickly. However, kaempferol, which also has a short half-live, was detected in a high number of serum samples. Kaempferol is found in a large number of fruits and vegetables, therefore, it is likely that women were exposed to a consistent small dose throughout their day. Whilst the xenoestrogen detected are all potential risk factors for breast cancer, since they all are capable of binding to ERs, it is very difficult to determine the magnitude of a specific exposure because of inter-individual variability (see section 7.4.7.).

Table 7.26: Biological half-lives of xenoestrogens studied

Compound	Approximate biological half-life (h)	Reference
BPA	2.0	(Stahlhut, *et al.*, 2009)
Kaempferol	2.8	(Wang, *et al.*, 2003)
Curcumin	6.5	(Jager, *et al.*, 2014)
Estriol	7.5	(Kuhl, 1990)
Genistein	8.2	(Chang, *et al.*, 2013)
Daidzein	9.5	(Chang, *et al.*, 2013)
Estrone	12.0	(Wecker, *et al.*, 2009)
Tetrahydrocurcumin	13.5	(Saradhi, *et al.*, 2010)
E2	16.9	(Zimmermann, *et al.*, 1998)
Butylparaben	24.0	(Abbas, *et al.*, 2010)
Benzylparaben	24.0	(Abbas, *et al.*, 2010)
Methylparaben	<24.0	(Abbas, *et al.*, 2010)
EE2	26.1	(Baumann, *et al.*, 1996)

7.4.1. Limitations

There are many complicating factors to consider in this exposure assessment, which made attempting to predict the effects of xenoestrogen exposure cocktails almost impossible. For example, polymorphisms in metabolism may mean that xenoestrogens have different half-lives depending on the person's ability to metabolise them. Therefore, simplifications and assumptions were applied to the calculation in order to predict and calculate the effect of xenoestrogen exposure cocktails. These included assuming the xenoestrogens were 100% absorbed, evenly distributed in the blood and not metabolised. Interestingly, even with these simplifications and assumptions, it is plausible that exposure to xenoestrogens could account for a substantial increase in total estrogenic load. In addition, this assessment has only addressed the estimated xenoestrogen exposure in terms of additivity; however, it is clear from previous experimental work (e.g. Chapters 4, 5 and 6) that there are many co-operating and competing factors at play. Therefore, the calculated total estrogenic effect is only theoretical. On the other hand, there are many more xenoestrogens that could not be accounted for in the study, and with the list of xenoestrogens continually growing, it is likely the theoretical total estrogenic effect is an underestimate of what women are exposed to daily.

7.4.2. Impact of Xenoestrogen Exposure on Total Estrogenicity

7.4.2.1. Women

This is based on the findings from Group 1 because they were a random sample of women from Canterbury. The percentage contribution of xenoestrogen exposures to the total estrogenic loads for Group 1 are shown in Table 7.27. On average xenoestrogens were calculated to contribute 51.3% to the total estrogenic loads of women, and in the WCS an

80.0% was calculated. This calculated xenoestrogen exposure could considerably increase the total estrogenic loads of women, potentially at least doubling normal circulating endogenous E2 levels. Whilst an approximate 51.3% contribution is not observed in all subgroups, even a small contribution (e.g. 9.6% in the 18-29 year subgroup) significantly increases the estrogenic potency of the blood in women. However, the postmenopausal subgroups of women experience a much higher contribution of xenoestrogens to their total estrogenic loads; between 61.5% and 77.3%. Not only does this mean that xenoestrogen exposure is at least doubling the estrogenic potency of their blood, but also that over 61.5% of the estrogenicity is not controlled by estrogen-mediated feedback mechanisms. Therefore, the only way to 'switch off' that portion of estrogenicity is to rely on metabolism (e.g. glucuronidation) (Liehr*, et al.*, 1998); thus, postmenopausal women are likely to be more susceptible to ER-mediated xenoestrogenic effects.

Table 7.27: Percentage contribution of xenoestrogens to the daily estrogenic loads of women from Group 1.

	Average %	WCS %
18-29	9.57	59.1
30-39	13.8	77.8
40-49	13.8	77.8
50-59	61.5	97.1
60-69	77.3	98.7
All	51.3	80.0

In terms of breast cancer development, the estimated increase in blood estrogenic potency observed in Canterbury women could lead to considerable amplification of the promotion and progression stages (Safe, 1998). Indeed, an increase in estrogenic potency remarkably increases the proliferation power of a breast cancer cell - a hallmark characteristic of cancer promotion and progression (Fouad*, et al.*, 2017). In postmenopausal women this increase in estrogenic potency (e.g. 67.6%), in conjunction with reduced biological control over the total estrogenic load, may partly explain why they are most at risk of developing breast cancer.

7.4.2.2. Pre-pubertal Girls

Both groups of girls were not randomly selected, therefore, they cannot be assumed to represent the wider population of pre-pubertal girls; thus, the two groups were combined for analysis. However, even though the daughters (Groups 1 and 2) and women (Group 1) were obtained using different sampling strategies, the findings were similar between the groups. This may mean xenoestrogen exposures could have biological implications (e.g. precocious

puberty, increased risk of breast cancer development post-menopausally) for pre-pubertal girls in Canterbury. The percentage contribution of xenoestrogen exposures to the total estrogenic loads for Group 1 are shown in Table 7.28. A good agreement between the percentage contribution of xenoestrogen exposure to the average total estrogenic load (58.1% and 63.5%) was observed; however, poorer agreement was observed between the calculated WCS total estrogenic loads (58.7% and 83.64%). This suggests that on average both groups of pre-pubertal girls have similar food and lifestyle habits but not at the WCS level. The younger (e.g. <6 years) subgroups of pre-pubertal girls were potentially more susceptible if exposed to xenoestrogens compared to the 11-14 year subgroup. This is likely because of their higher food intake per body weight, and lower circulating E2 levels, so that the contribution of xenoestrogens is higher for these subgroups (Aksglaede*, et al.*, 2006). Interestingly, these same subgroups of pre-pubertal girls experienced, at a minimum, an 83.0% contribution from xenoestrogen exposure to their total estrogenic loads. It is important, but highly complex, to consider the relatively estrogenicities of xenoestrogens in a mixture because this will significantly affect the sum of their interactions with the ERs. I did not attempt to investigate this but clearly this warrants further study. This is a significant increase in the estrogenic potency of their blood; therefore, they are likely to be more susceptible to ER-mediated xenoestrogenic effects compared to the older pre-pubertal girls.

Table 7.28: Average of the percentage contribution of xenoestrogens to the daily estrogenic loads of pre-pubertal girls from Groups 1 and 2. Errors are expressed as SEM.

	Average %	SEM	WCS %	SEM
0-12 moths	83.9	0.0	99.6	0.0
1-3 years	92.8	6.3	99.3	0.2
5-6 years	85.7	2.6	92.8	4.6
11-14 years	52.3	0.4	69.1	7.3
All	60.8	2.7	71.2	12.5

Not only is breast cancer complex, but so is breast development. Breast tissue consists of multiple cell types, which must remain in close communication. Normal mammary gland growth involves intricate crosstalk between the epithelium and surrounding stroma, to balance proliferation/apoptosis and remodel the gland at the different stages of life (see Fig. 1.28) (Macon*, et al.*, 2013). Exposure to xenoestrogens during this highly susceptible time could affect mammary gland development, potentially increasing susceptibility to chemical carcinogens, and in turn, breast cancer development later in life. The increase in estrogenic load calculated from these studies clearly demonstrates the potential to affect mammary gland development with the high potential proliferative power of circulating xenoestrogens

and limited biological controls (Liehr, *et al.*, 1998) in pre-pubertal girls. However, cause-effect relationships between pre-pubertal xenoestrogen exposure and increased breast cancer risk later in life are yet to be established (Rodgers, *et al.*, 2018).

On the other hand, the pre-pubertal girls could also be more susceptible to other adverse health effects, such as precocious puberty (Massart, *et al.*, 2006). Precocious puberty refers to the appearance of physical and hormonal signs of pubertal development at an earlier age (e.g. 8 - 10 years in girls (Carel, *et al.*, 2004)) than is considered normal. The release of E2 in the body, which is a result of increased levels of gonadotrophins, is responsible for initiating the process of puberty naturally (Mueller, 2004). Therefore, exposure to xenoestrogens could initiate this process much earlier than expected. Indeed, a study which examined chronic xenoestrogen exposure observed early signs of pubertal development in mice in the absence of normally increased levels of gonadotrophins (Massart, *et al.*, 2006). This suggests that xenoestrogens could circumvent the natural pubertal control mechanisms, inducing puberty at an earlier age. In this study the calculated xenoestrogen exposures for the subgroups of pre-pubertal girls increased the calculated total estrogenic load. Therefore, the pre-pubertal girls included in the study could also be susceptible to precocious puberty. Indeed, the general trend of age of menarche (e.g. first period) around the world is decreasing (Fig. 7.8) and xenoestrogen exposure could be involved in this decrease.

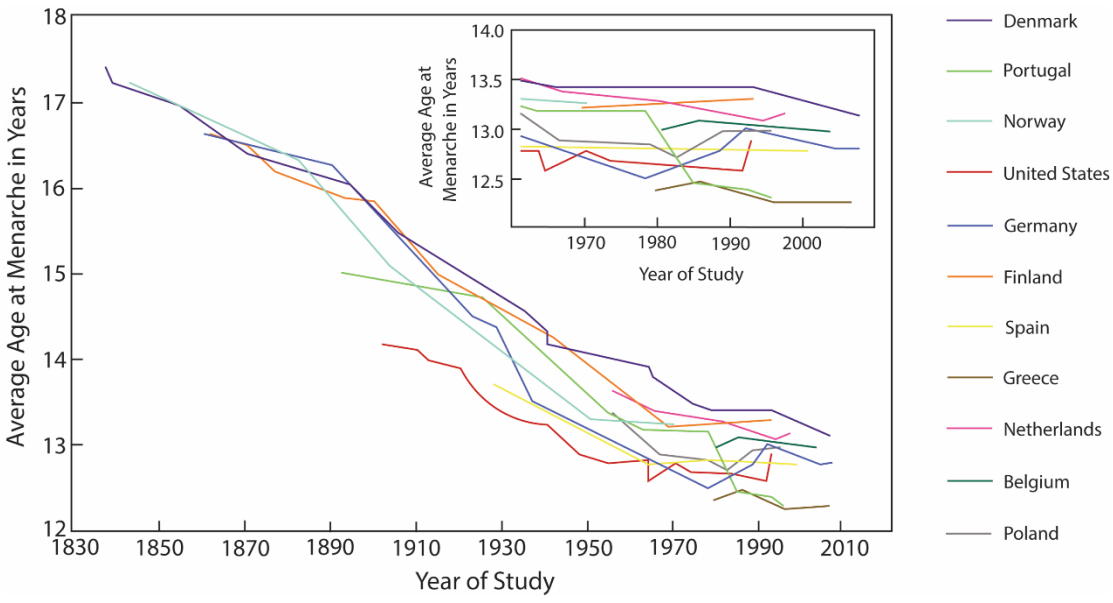


Figure 7.8: Trends in age of menarche (Sorensen, *et al.*, 2012).

Whilst precocious puberty is not often directly associated with an increased breast cancer risk, it is well established that the length of time between puberty and a woman's first full term pregnancy (e.g. parity) is associated with an increased risk of breast cancer (Kobayashi, *et al.*, 2012). Therefore, decreasing the age of puberty due to xenoestrogen exposure, and potentially increasing the age between puberty and parity, could be increasing breast cancer risk. Interestingly, it has been shown that an approximate 20% decrease in breast cancer risk results from each year of delayed menarche (Feigelson, *et al.*, 1996). On the other hand, earlier menarche also means women are able to have children younger (Forman, *et al.*, 2013). Whilst the average age for women having their first child is increasing (Shadyab, *et al.*, 2017), there is also a decrease in the age at which some women are having children (i.e. because of earlier menarche) (Forman, *et al.*, 2013). This, however, also creates a gap between biological and social maturity in some societies, with individuals becoming biologically adult at earlier ages but remaining minors socially and legally (Volgyi, 2008). Therefore, girls who are biologically mature are faced with considering the implications of environmental exposures, such as the impact of xenoestrogens, which could impact their health later in life; yet these girls are unlikely to be mature enough to consider these implications. Therefore, the possible impacts of xenoestrogens may be more far reaching than previously anticipated.

7.4.3. Comparison of Total Estrogenic Loads of Women and Pre-pubertal Girls

Interestingly, there is a high degree of commonality between women (Group 1) and pre-pubertal girls as shown in Table 7.29. As discussed above, postmenopausal women and pre-pubertal girls are likely to be most susceptible to the adverse effects of xenoestrogen exposure. Postmenopausal women are the most likely group of women to develop breast cancer, not only because of the high contribution of xenoestrogens to their total estrogenic loads, but also, they are the most susceptible to other risk factors, especially age; thus, they are more susceptible to cellular transformation (e.g. initiation). The other group of participants that are likely to be the most susceptible to the adverse effects of xenoestrogen exposure are pre-pubertal girls. Whilst this potential exposure is unlikely to result in an immediate breast cancer diagnosis (e.g. within 5 years), it could increase the risk of breast cancer development later in life. Indeed, for at least two other known breast cancer risk factors (e.g. radiation and migration (Carpenter, *et al.*, 2013, Ziegler, *et al.*, 1993)) it appears that exposure to these risk factors during crucial developmental stages (e.g. pre-puberty) can translate an increase in breast cancer diagnoses later in life; thus, exposure to xenoestrogens during this window of susceptibility could increase breast cancer incidence for these pre-

pubertal girls. In addition, women of child-bearing age are potentially not as susceptible if exposed to xenoestrogens compared to the other groups of postmenopausal women and pre-pubertal girls; however, they are susceptible to breast cancer initiation. This susceptibility is likely a combination of other risk factors including exposure to carcinogens, and high circulating endogenous E2 levels (Feuer*, et al.*, 1993).

Table 7.29: Comparison of average total daily xenoestrogen exposure between women and pre-pubertal girls.

Age	Average contribution (%) in women (Group 1) and pre-pubertal girls
Women	
18-29	9.6
30-39	13.8
40-49	13.8
50-59	61.5
60-69	77.3
All ages (18 18-69 years)	51.3
Pre-pubertal girls	
0-12 months	58.1
1-3 years	83.9
5-6 years	86.5
11-14 years	83.1
All ages (0-14 years)	51.9

7.4.4. Impact of Hormonal Medications

Strong commonality was observed for the percentage contributions of hormone-based medicines to the total estrogenic loads between Group 1 subgroups (Table 7.30). This is not surprising given the high concentrations (e.g. $5.1 \times 10^{-7} – 8.7 \times 10^{-7}$ M in hormone-based contraceptives) of estrogens present in these medicines, which essentially overwhelm any endogenous E2 present. The xenoestrogen exposures appear to be a ‘drop in the bucket’ compared to the hormone-based medicines; therefore, they have a small contribution to the calculated total estrogenic loads. Thus, the estrogen-mediated biological effects of these medicines could have serious implications on human health – such as is the likely case for breast cancer risk. Hormone-based contraceptives do increase breast cancer risk; however, this increase is small and in a population of women that already have a low risk the increase in risk from hormone-based contraceptives is often regarded as negligible (Morch*, et al.*, 2017). On the other hand, postmenopausal women a small increase in risk from HRT medicines could translate into a lot more women developing breast cancer. The calculated exposures for hormone-based contraceptives presented here clearly demonstrate the significant increase in estrogenic potency of blood. This increase likely increases the

proliferative power of the circulating xenoestrogen which could arguably drive the promotion and progression stages of breast cancer. Indeed, a recent Danish study found that breast cancer risk was higher among women who were currently taking or had recently used estrogen contraceptives compared to women that had never used them (Morch*, et al.*, 2017). However, a definite link between hormone contraceptives and breast cancer still remain to be established.

Table 7.30: Comparison of the percentage contribution of xenoestrogens to the daily estrogenic loads of women using hormone contraceptives for Group 1 subgroups.

Participants	Average xenoestrogen contribution (%)	WCS xenoestrogen contribution (%)
18-29	96.6	97.1
30-39	96.5	96.9
40-49	96.6	96.5
50-59	99.7	99.7
60-69	100.0	99.5
All	96.9	90.6

In addition, HRT medicines increase the total estrogenic load 100-fold compared to hormone-based contraceptives, ranging from $1.6 \times 10^{-5} – 5.1 \times 10^{-5}$ M (Table 7.31). Interestingly, but not surprisingly, HRT use in this study was limited, with no women from Group 2 using these medicines, perhaps on the account of the widespread knowledge about the link between HRT use and increased breast cancer risk (Rossouw*, et al.*, 2002). The significant increase in total estrogenic load calculated from these studies clearly demonstrates the high potential proliferative power of the circulating exogenous estrogens. In contrast to other xenoestrogens (e.g. phytoestrogen, parabens, etc.), these medicines are likely to be controlled by estrogen feedback mechanisms. This especially applies to HRT medicines which often consist of E2 isolated from external sources (e.g. equine urine). Therefore, this could reduce the potency of these medicines on breast cancer promotion and progression. Conversely, women who generally use HRT medicines are postmenopausal; thus, they likely have a low endogenous E2 production to begin with. Therefore, the reduction in potency due to estrogen feedback control mechanisms is likely to be negligible in comparison to the proliferative power of the medications.

Table 7.31: Percentage contribution of HRT to the total estrogenic load for Group 1.

	Average %	WCS%
18-29	0.0*	0.0
30-39	0.0*	0.0
40-49	99.9+	99.9
50-59	100.0#	100.0
60-69	100.0#	100.0
All	99.9+	99.9

* This age group does not receive HRT; + represents a 1000 fold increase; # the natural estrogen levels in this age-group are incredibly low therefore, it was not possible to calculate a fold increase.

E2 is classified as a carcinogen by the International Agency for Research on Cancer (IARC). It gained this classification based on data that suggest "E2 is a weak carcinogen and mutagen capable of inducing genetic lesions with low frequency" (Liehr, 2000). It is thought E2 initiates tumour formation by the metabolic conversion of E2 to 4-hydroxyestradiol, which is further activated by catechol to catechol estrogens which are hydroxyquinones. These can be readily oxidised to DNA-reactive semiquinone/quinone intermediates (Fig. 7.9; see Section 1.5.3.) (Liehr, 2000). Therefore, HRT medicines are essentially a double-edged sword in the context of breast cancer development – inducing DNA damage, which is characteristic of the initiation stage, and promotion and progression of tumour growth via ER-mediated mechanisms. Hence, it is not surprising that women are at a higher risk of developing breast cancer when using HRT medicines.

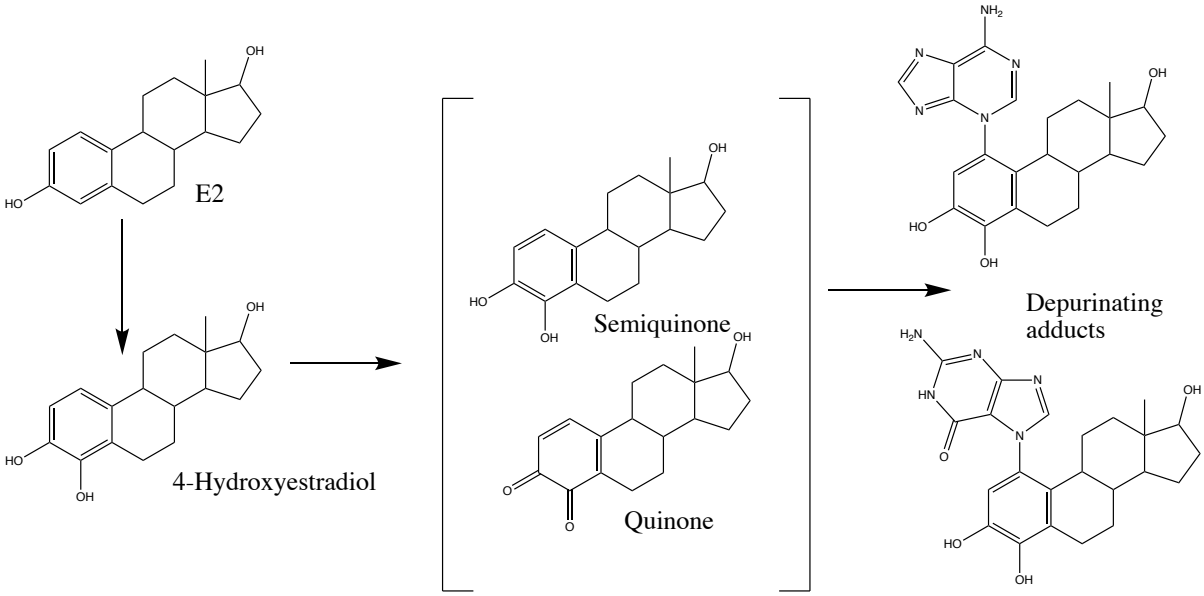


Figure 7.9: Major metabolic pathway of quinone/semiquinone production from E2

7.4.5. Impact of dietary supplements

The term 'dietary supplements' once analogous with vitamins and minerals, is now used to include botanical supplements such as echinacea, various forms of garlic and a variety of physiologically active compounds derived from foods or other biological materials. Although there has been a worldwide trend of an increase in the prevalence of dietary supplement use (Briefel*, et al.*, 2004, MacLennan*, et al.*, 2002, Tindle*, et al.*, 2005), the necessity for, and cost and safety of, dietary supplements continues to be debated (Radimer, 2005). The results presented here found dietary supplements were commonly used by women, with 37.4% of participants taking at least one form of supplement. Interestingly, dietary supplement use significantly increased for the older subgroups with over 75% of participants between the ages of 40-69 years. In a 2006 New Zealand study on dietary supplement use, 30.5% of women and 5.4% of children were taking a dietary supplement (Parnell*, et al.*, 2006). The results presented in this chapter are consistent with these findings for women; however, on average 20.9% of pre-pubertal girls were found to be taking at least one form of dietary supplement (Table 7.32) – almost quadruple of what was reported in the 2006 study. This may be a result of multiple variables between the studies including the non-random sampling of pre-pubertal girls in this study, dependence on whether their mother was also taking a dietary supplement, the time of year the data were collected (e.g. people are more likely to take supplements such as vitamin C in the winter), and that only girls were included in this study compared to the 2006 study which also included boys.

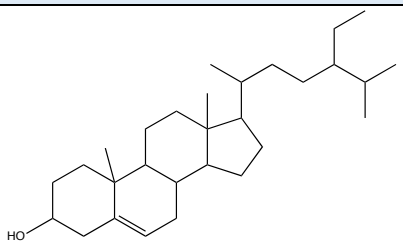
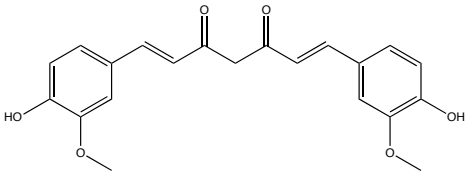
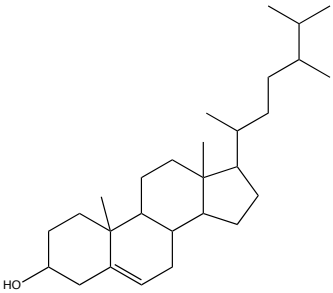
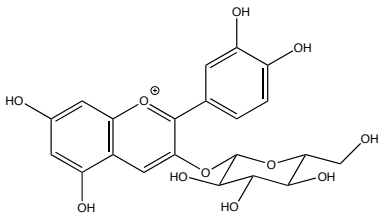
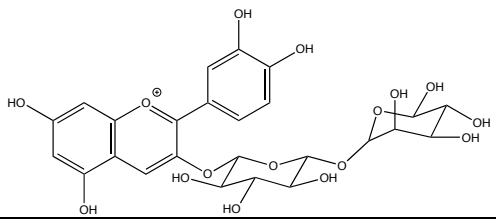
Table 7.32: Average number of participants whose pre-pubertal daughters are consuming dietary supplements from both groups.

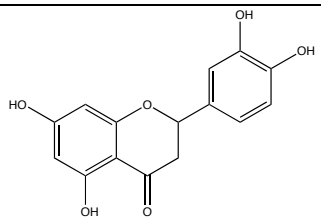
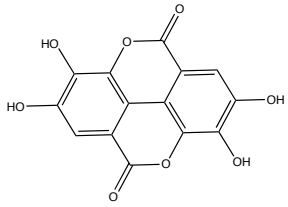
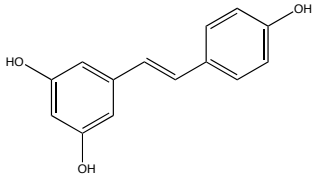
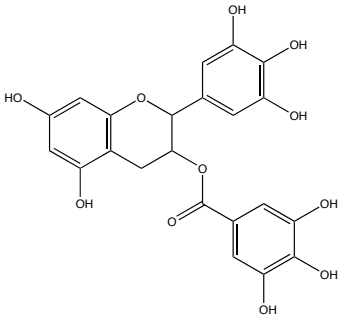
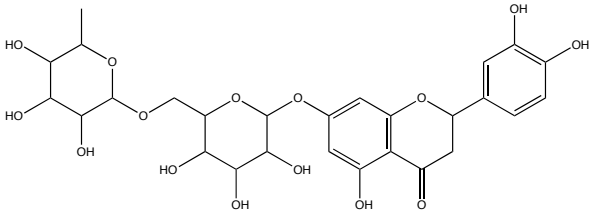
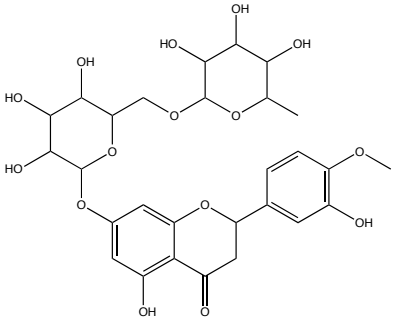
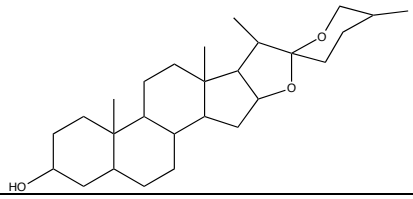
	Range of number of positive responses returned (%)	Average (%)
0-12 months	0	0.0
1-3 years	0	0.0
5-6 years	0 - 50	25.0
11-14 years	0 - 20	10.0
Total	11.1 – 30-6	20.9

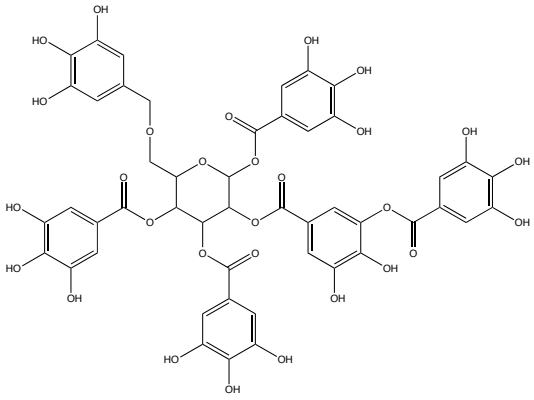
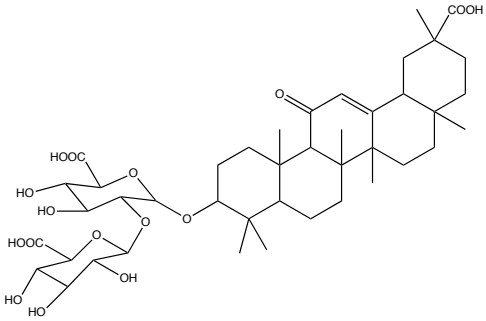
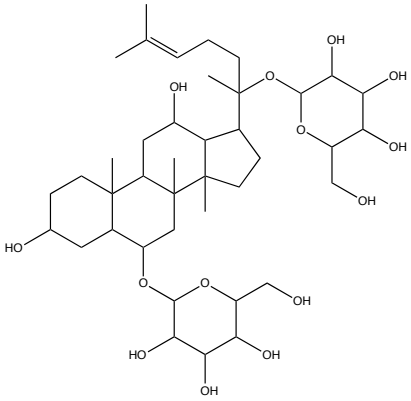
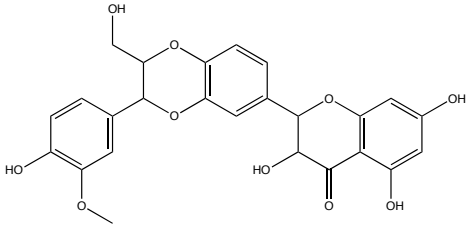
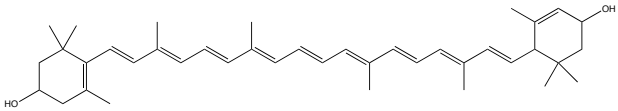
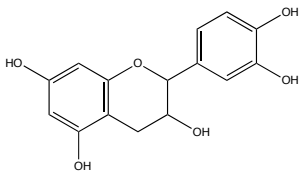
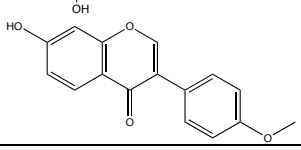
Interestingly, dietary supplements could be one of the biggest sources of phytoestrogens; however, people may not consider them as risk factors for breast cancer. Upon closer analysis of the dietary supplements taken by women and pre-pubertal girls in this study, it is clear there are a multitude of phytoestrogens present in the dietary supplements (Table 7.33). This

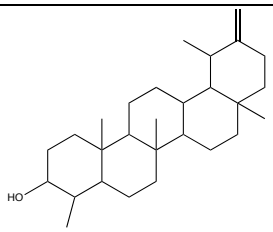
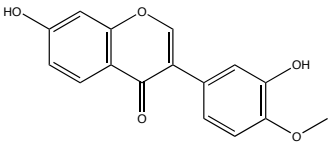
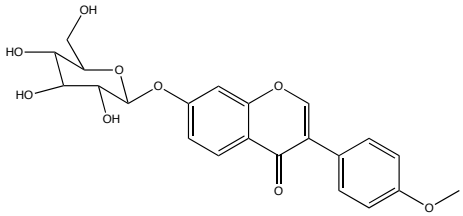
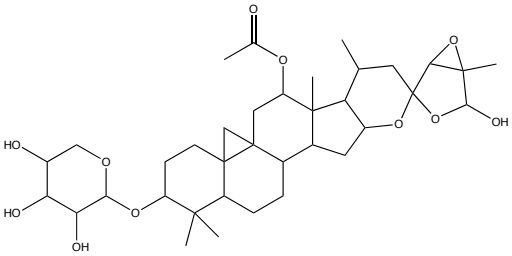
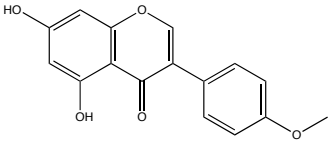
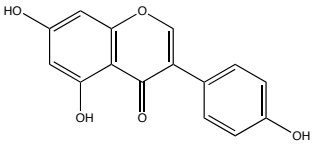
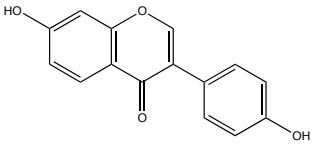
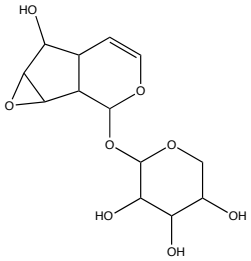
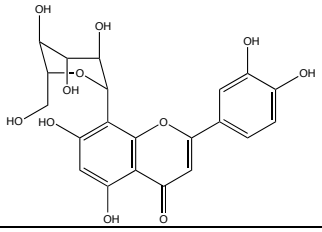
presents a very complex picture when considering dietary supplements in the context of total estrogenic load, and therefore, attempting to predict total estrogenicity is almost impossible. In addition, there is limited information available on the levels of phytoestrogen in dietary supplements; therefore, it was not possible to include them in the calculated total estrogenic loads. However, when comparing the structures of the potential phytoestrogens in the dietary supplements to E2, it is likely that they would elicit an estrogenic effect based of on their structure activity relationship (SAR) (Table 7.33). Therefore, dietary supplements are often estrogenically active which could potentially contribute to the promotion and progression stages of breast cancer; thus, potentially adding to breast cancer risk.

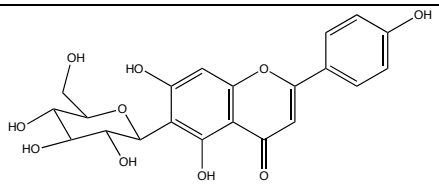
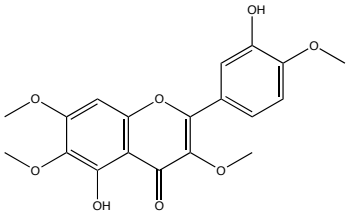
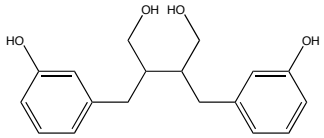
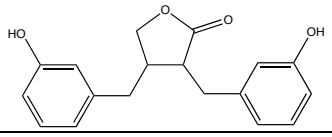
Table 7.33: List of structures of phytoestrogens found in dietary supplements consumed by study participants.

Phytoestrogen	Plant source	Structure
β-Sitosterol	Evening primrose, dong quai, 5 flavour berry and goji	
Curcumin	Tumeric	
Campesterol	Evening Primrose	
Cyanidin 3-glucoside	Acai	
Cyanidin 3-rutinoside	Acai and celery	

Quercetin	Rosehips and ginkgo	
Ellagic acid	Rosehips	
Resveratrol*	Grape seed oil	
Epigallocatechin gallate	Green tea	
Eriocitrin	Lemon	
Hesperidin	Lemon	
Saporin	Papaya	

Tannin	Papaya, celery, black cohosh and feverfew	
Glycyrrhizin	Licorice	
Ginsenoside	Ginseng	
Silymarin	St Mary's thistle/milk thistle	
Lutein	Artichoke	
Catechin	Ginkgo	
Formononectin	Huang qi and red clover	

Taraxasterol	Dandelion	
Calycosin	Huang qi	
Ononin	Huang qi	
Acetin	Huang qi and black cohosh	
Biochanin A	Red clover	
Genistein	Soy and red clover	
Daidzein	Soy and red clover	
Catapol	Rehmannia	
Orientin	Vitex (Chase tree)	

Isovitexin	Vitex (Chase tree)	
Casticin	Vitex (Chase tree)	
Enterodiol	5 flavour berry (protein powder)	
Enterlactone	5 flavour berry (protein powder)	

* Resveratrol is also found in wine and other foods; however, this table is only considering the dietary supplements women in the studies indicated they were taking.

7.4.6. Relationship between Breast Cancer and Xenoestrogen Exposure

7.4.6.1. Breast Cancer Diagnosis

In this study of women aged 18-69 from Group 1 (a population-based sample from the electoral roll), 6.0% of participants had been diagnosed with breast cancer at the time of the study. For the 50-59 and 60-69 subgroups 9.6% of participants had been diagnosed with breast cancer at the time of the study. This is not surprising as breast cancer is the most prevalent in post-menopausal women. However, 3.9% of the 18-29 and 30-39 subgroups had been diagnosed with breast cancer. This equates to 14.3% of the total number of breast cancer diagnoses and is above the 6% average number of women diagnosed with breast cancer under the age of 40 years in New Zealand. On the other hand, 70%-75% of women diagnosed with breast cancer are over the age of 50 years in New Zealand (NZBCF, 2018). The proportion of women in this study that were over the age of 50 were 85.7%. Therefore, the distribution of women for the under 40-year-old and over 50-year-old women were slightly higher than the national averages; however, suggest that both groups were representative of the New Zealand population. Women with breast cancer may be over-represented in the study sample because their diagnosis may have increased their willingness to participate.

7.4.6.2. Consideration of Xenoestrogens as Breast Cancer Risk Factors

Surprisingly, 32.2% of women from Group 1 had considered xenoestrogens as breast cancer risk factors prior to participating in the study; while 44.5% of women with a pre-pubertal daughter had considered xenoestrogens as breast cancer risk factors for their daughter (Table 7.34). It is remarkable that such a high percentage of women had considered xenoestrogens as breast cancer risk factors, suggesting that women are much more aware of this potential link than anticipated. In general, the percentage of women that had considered xenoestrogens as breast cancer risk factors was similar across subgroups; from 35.5% of the 18-29 subgroup to 33.3% of the 60-69 subgroup. This is a surprising knowledge but again might reflect their interest in the study. Interestingly, the 50-59 subgroup did have a lower awareness, with 26.7% of the subgroup considering xenoestrogens as breast cancer risk factors.

Table 7.34: Study average of women who had considered xenoestrogens breast cancer risk factors for their daughters (Group 1 and 2) prior to participating in the study.

	Range of number of positive responses returned (%)	Average (%)
0-12 months	66.7	66.7
1-3 years	25.0 – 50.0	37.5
5-6 years	16.7 – 100.0	58.4
11-14 years	60.0 – 66.7	63.35
Total	35.3 – 53.7	44.5

7.4.6.3. Elimination of Xenoestrogens from Daily Food and Lifestyle Habits

Whilst 32.2% of women from Group 1 had considered xenoestrogens as breast cancer risk factors, only 30.0% had taken measures to eliminate them from their daily food and lifestyle habits. This illustrates that while information may be available to women on xenoestrogens and breast cancer risk, it is only as successful as the person who is willing to change their lifestyle. Therefore, one could argue that the most important aspect of encouraging women to make lifestyle changes, that could reduce their risk of breast cancer, is not only providing them with the information but rather creating a positive mindset equipping them to apply this information. In this study, the difference between women who had considered xenoestrogens as breast cancer risk factors and those that had followed through to eliminate them from their daily food and lifestyle habits is approximately 7.0%. This suggests that a majority of women are prepared to create changes in their food and lifestyle habits to reduce breast cancer risk, which could have a major follow on effect in reducing the breast cancer burden in New Zealand.

In comparison, 45.9% of women who completed a questionnaire for their pre-pubertal daughter had taken steps to eliminate xenoestrogens from her daily food and lifestyle habits (Table 7.35). This is an increase in the percentage of women who considered xenoestrogens as breast cancer risk factors for their daughter, demonstrating that they may be eliminating xenoestrogens (e.g. BPA plastics) for other reasons than breast cancer risk.

Table 7.35: Study average of women who had taken steps to eliminate xeneostrogens from their daughter's (Group 1 and 2) daily food and lifestyle habits prior to participating in the study.

	Range of number of positive responses returned (%)	Average (%)
0-12 months	66.7 -	66.7
1-3 years	25.0 -	37.5
5-6 years	16.7 -	58.4
11-14 years	60.0 -	63.35
Total	38.2 -	45.9

Interestingly, when women were asked to specify the measures they had taken to eliminate xenoestrogens from their daily habits, almost all women (95%) had eliminated BPA plastics and/or paraben containing cosmetics and personal care products. This clearly demonstrates a public awareness (e.g. BPA-free containers and paraben-free cosmetics) of the synthetic xenoestrogens and potential breast cancer risk; however, very few women (<5%) had eliminated natural xenoestrogens (e.g. phytoestrogens). This may be a result of the controversial effects of phytoestrogens (e.g. at low concentrations they increase breast cancer risk and at high concentrations are protective against breast cancer) or a clear bias of public perception towards synthetic chemicals being risker than natural chemicals. Indeed, BPA has been specifically regulated in the use of manufacturing babies bottles in many countries (Almeida*, et al.*, 2018, JRC/IHCP, 2010); this could have a significant impact on public perception of xenoestrogens. Phytoestrogens have unique estrogenic potencies, which makes understanding phytoestrogens in a breast cancer risk context incredibly complex – it is not as simple as eliminating them from daily food habits. Therefore, it is not surprising women have chosen to focus on the synthetic xenoestrogens which appear to be less complex; however, it must not be forgotten that the breast cancer risk of the synthetic xenoestrogens can only be determined in the context of the total risk of phytoestrogens from food. Thus, an

understanding of the dual effects of phytoestrogens is an important in attempting to create new strategies for reducing breast cancer risk.

7.4.7. Detection of Xenoestrogens in Group 2 Blood Samples

This qualitative analysis detected multiple xenoestrogens in the blood from women in Group 2, clearly showing that the xenoestrogens predicted to be present from the questionnaire analysis were found in the blood samples of the participants. The levels of xenoestrogens detected were low, characteristic of chronic exposure rather than acute high-level exposures, which are often associated with rapid increases and clearance profiles. Logically one would expect xenoestrogen exposures to be chronic considering many products which contained xenoestrogens are used/consumed daily, if not multiple times a day. Therefore, it is reasonable to assume that the exposures to xenoestrogens are chronic and low-level rather than an acute exposure (e.g. workplace exposure) (Graham, 2012). For example, studies on workplace exposure to BPA in supermarket cashiers clearly demonstrate this characteristic acute dosing profile. The workers experience a rapid increase in BPA in their blood when they started their shift that rapidly cleared once their shift finished approximately 8 hr later (Ndaw*, et al.*, 2016). On the other hand, if the exposure is very frequent, even though the half-life of the compound is short, one could get a pseudo-steady state level. However, in this study it is much more likely that exposure to xenoestrogens is intermittent which would lead to peaks and troughs. This could have affected the detection of some xenoestrogens in the samples.

7.4.7.1. BPA

BPA was not detected in any of the 47 blood samples analysed. This was not unexpected as BPA is metabolised very rapidly (~2 hr) (ref), which makes the chances of detection low. In addition, BPA has received a lot of negative public attention, leading to a massive reduction in the use of BPA containing plastic products. Therefore, in conjunction with the rapid metabolism, the lower exposure likely has led to BPA not being detected in the study.

7.4.7.2. Parabens

Methyl- and butylparaben were the most commonly detected xenoestrogens in the study, with 100% and 87.2% of samples detecting the parabens, respectively. This finding is not surprising, especially for methylparaben (see Section 1.4.3.), given the extensive use of parabens in cosmetics and personal care products. In addition, parabens generally have long half-lives (approximately 24 h; (Abbas*, et al.*, 2010)) in comparison to other xenoestrogens

(e.g. genistein which has a half-life of 8.2 h; (Chang, *et al.*, 2013)). Interestingly, methylparaben has been reported to exhibit only a 5% degradation over 24 h in human serum (Abbas, *et al.*, 2010). Therefore, the longer half-lives are likely contributing to the common detection of methyl and butylparaben. Benzylparaben was only detected in one of the 47 blood samples (2.1%); however, it is not as commonly used in cosmetics and personal care products. Therefore, it is not surprising to find that it was only detected in 2.1% of samples. Interestingly, parabens were the second most common xenoestrogen, next to BPA, that women eliminated from their daily food and lifestyle habits; however, they were detected in every blood sample. This illustrates the persistence of parabens but also the potential gaps in knowledge of how widespread paraben use is in personal care products and cosmetics. On the other hand, in this thesis butylparaben has been demonstrated to have similar effects to phytoestrogens in breast cancer model systems (e.g. MCF-7 and CALUX® studies); thus, the overall estrogenicity of parabens, in particular butylparaben, may not be as potent as it is currently considered. This is certainly something to deliberate when attempting to implement new breast cancer prevention strategies by changing food and lifestyle habits.

7.4.7.3. Phytoestrogens

Phytoestrogens are widely distributed in plants and are found in many food plants (e.g. cruciferae, such as brussel sprouts, Rosaceae, such as apples). Therefore, it was not unexpected that phytoestrogens were the other group of xenoestrogens that were commonly detected in the blood samples. Genistein and daidzein were only detected in 34.0% and 29.8% of the blood samples, respectively. This is surprising given the widespread use of soy flour in baked goods (e.g. bread) and cereals (Rudel, *et al.*, 2009) and the relatively long half-lives of these isoflavones, 8.2 h and 9.5 h (Chang, *et al.*, 2013), respectively. This suggests that soy flour is being used less in baked goods or perhaps people are consuming less baked foods and cereals for personal health reasons (e.g. for a gluten intolerance or coeliac disease). Interestingly, kaempferol was detected in a much high proportion of blood samples (89.4%). It is found in a wide variety of foods including apples, grapes, tomatoes, green tea, potatoes, onion, broccoli, squash, blackberries, etc (Calderon-Montano, *et al.*, 2011, Kim, *et al.*, 2013, Liu, 2013); therefore, it is not surprising to find kaempferol in a majority of samples especially given its much shorter half-live (2.8 h; (Wang, *et al.*, 2003)) compared to other phytoestrogens. Interestingly, it is estimated that the total average daily intake of kaempferol is 3.91 mg (Liu, 2013). Some of the highest kaempferol containing foods include radish (32.3 mg/kg) and peas (62.8 mg/kg) (Cao, *et al.*, 2010). Curcumin and tetrahydrocucumin were detected in smaller percentages of blood samples, 27.7% and 2.0%, respectively. It was

unexpected to find tetrahydrocurcumin as it is one of 5 metabolites of curcumin (Tsuda, 2018); however, this is an important finding because tetrahydrocurcumin, as shown in Chapter 4, has anti-proliferative effects in MCF-7 cells. Because it was only detected in one blood sample, the likelihood of women consuming enough turmeric to then have sufficient levels of curcumin which then is metabolised to tetrahydrocurcumin is low; therefore, it is unlikely that eating turmeric would provide sufficient levels of tetrahydrocurcumin for the women in this study to obtain a beneficial effect.

7.4.7.4. Estrogens

E2 was detected in a high proportion of samples (68%), however, only 40% of the 18-29 subgroup had detectable levels of E2. This is surprising given the high fluctuating levels (9.9 x 10^{-11} – 1.6 x 10^{-9} M (Elmlinger*, et al.*, 2002)) of E2 during the menstrual cycle in women of this age group. In comparison, more women in the 60-69 subgroup had detectable E2 levels, even though they have lower circulating E2 levels (0 – 3.7 x 10^{11} M). This could suggest a higher exposure to exogenous E2 from an accumulation of dairy, meat and egg products for this subgroup. Indeed, previous studies have reported the presence of estrogens in these products (Hu*, et al.*, 2012, Malekinejad*, et al.*, 2015, Shahbazi*, et al.*, 2016). Neither estrone nor estriol were detected in any of the 47 blood samples. Again, this is not surprising given the lower levels of estrone (2.6 x 10^{-11} – 7.4 x 10^{-10} M (Cummings*, et al.*, 1998)) in women, which is likely below the limit of detection for the study. Estriol is the primary estrogen produced by the placenta during pregnancy; thus, since it was unlikely any of the participants were pregnant it was unlikely that estriol would be detected. EE2 was detected in one of the samples. This was not surprising given the participant was taking an EE2-containing hormone replacement therapy.

7.4.8. Are Xenoestrogens Breast Cancer Risk Factors?

This human exposure study shows that the predicted xenoestrogens from the questionnaire are found in the blood of the participants. The women are clearly exposed to xenoestrogens from their daily food and lifestyle habits. Importantly, this study highlights that women are not just exposed to one xenoestrogen, but rather to multiple xenoestrogens, with high inter-individual variability, which illustrates a highly complex xenoestrogen exposure cocktail scenario. This complexity makes understanding the xenoestrogen cocktail effect much more difficult, especially given the mixed proliferative/anti-proliferative effects of some xenoestrogens (e.g. genistein), and an impossibly difficult situation when it comes to potentially regulating xenoestrogens.

As mentioned above, women are exposed to multiple xenoestrogens. Despite different sampling techniques, there was a high degree of commonality between the groups of women, and between the women (Group 1) and pre-pubertal girl subgroups; therefore, one may expect Canterbury women and pre-pubertal girls to have similar circulating xenoestrogens - albeit with inter-individual variability. The estrogenic potency of these circulating xenoestrogens could effect the breast cancer promotion and progression stages, with the proliferative power of the xenoestrogens driving breast cancer cell growth. Indeed, all the xenoestrogens included in this study were previously demonstrated to have ER LBC binding capabilities (see Chapter 6); therefore, it would be expected that they would drive proliferation which could lead to tumour growth. Thus, based on the calculated xenoestrogen exposures and the qualitative blood analyses, Canterbury women could have an increased risk of developing breast cancer.

7.5. Concluding Remarks

Exposure to xenoestrogens is a life-long health risk where the effect may be greater later in life because of lower levels of endogenous estrogens and thus, lack of homeostatic control. Complex exposures, as demonstrated in this chapter, may play a role in the increase of breast cancer incidence, with certain lifetime windows associated with an increase in risk. However, simply avoiding xenoestrogens is not necessarily the best way to reduce breast cancer risk. Indeed, it is clear that even when women intentionally avoid parabens that methylparaben and/or butylparaben are still present in the blood (see Section 7.4.6.2.). On the other hand, phytoestrogens have clearly been demonstrated to exert anti-proliferative effects in breast cancer cells in this thesis, thus eliminating natural xenoestrogens may be counterintuitive. Therefore, much more research is required to truly understand the complexity of xenoestrogens cocktails as breast cancer risk factors.

Chapter 8 Overall Discussion

8.1. Overall Discussion

Since the first inkling that exogenous molecules could interfere with receptor-based sex steroid hormone activity in the early 1990's (Muller, *et al.*, 1995) through to the discovery of xenoestrogens in the late 1990's (Colborn, *et al.*, 1996, Hunter, *et al.*, 1993) and our more recent detailed understanding of the complex workings of ERs, many millions of dollars have been spent on research to understand the impact on human populations. This represents a huge world effort to understand endocrine disruption; in particular estrogen mimicry and its impact on ecosystems and humans alike. These impacts are potentially profound as is evidenced by declining human sperm counts around the world (Carlsen, *et al.*, 1992, Levine, *et al.*, 2017, Swan, *et al.*, 2000), precocious puberty in girls (Massart, *et al.*, 2006), vitellogenin expression in male trout (Kidd, *et al.*, 2007, Sumpter, *et al.*, 1995), disruption of breeding cycles in sea bass (Blazer, *et al.*, 2012) and skewed sex ratios in some molluscs (Davies, *et al.*, 1997), etc., etc.

As we grapple with the complexity of xenoestrogens' impact on human populations, it is becoming clearer that they might have far reaching effects beyond those originally conceived. We now know that E2 is not just a female hormone but plays a key role in growth and developmental processes, particularly at the embryonic stage (Stocco, 2012, Vasquez, *et al.*, 2013). This means that xenoestrogens' effects are likely to reach beyond control of female physiology and biochemistry and extend into growth and developmental phenomena; this might have significant implication, in a long-term exposure context, for human growth and development and the aetiology of disease. Indeed, xenoestrogens are emerging health risk factors; they are being increasing investigated as potential risk factors for developmentally-based diseases such as breast cancer – the underlying subject of this thesis.

Concerns about the potential for environmental chemicals, drugs, and other stressors to alter endocrine physiology have quickly mounted and received a great deal of attention within the toxicological community as well as in the public media (Marty, *et al.*, 2011). Xenoestrogens have come to the forefront of toxicology over the last 20 years, with prominent government and scientific organisations such as the WHO, International Union of Pure and Applied

Chemistry (IUPAC) and the US National Research Council reacting to gather endocrinologists, toxicologists, and other public health professionals from around the world to capture their knowledge and recommend research to address this issue. These efforts are documented in a series of comprehensive review papers and reports (Damstra*, et al.*, 2002, Lintelmann*, et al.*, 2003, NRC, 1999, UNEP/WHO, 2013), which helped spur on endocrine-mediated toxicology. The concern at a government level over the possible effects of xenoestrogens is also reflected in the millions of dollars allocated by European and American agencies to support research on xenoestrogens as endocrine disruptors; for example, $23 M by the European Union in 2003 (Lorenz, 2003). Clearly governments are concerned about the lasting effects of these chemicals on their populations. This research is wide ranging and includes, for example, food safety of phytoestrogens, the screening of many thousands of chemicals for estrogenic effects, how these chemicals interact with one another and how they influence gene expression. However, the precise impact of xenoestrogens is difficult to discern as they can be both beneficial and adverse, producing different effects in different tissues at different life stages (Marty*, et al.*, 2011).

The effects of xenoestrogens are not simple. Individual compounds (e.g. genistein) can have differential biological effects at different exposure levels; for example, at 'low' exposure concentrations genistein promotes MCF-7 cell proliferation in culture, whereas at 'high' concentrations it inhibited growth (see Chapter 4). This means that the effects xenoestrogen risk factors are dose dependent and difficult to predict without a significant understanding of their complex interactions with ERs' ligand binding sites. This thesis addresses these issues at both the biological activity and *in silico*-modelled receptor interaction level.

To make the human xenoestrogen exposure scenario even more complex, we are exposed to a constant and constantly varying cocktail of xeneoestrogens via food (e.g. genistein in soy), by direct interaction with our environment (e.g. pyrethroid pesticide residues) and via personal care products (e.g. butylparaben in cosmetics). Since xenoestrogens all interact with the ERs, their effects are likely at least additive – this is a major focus of this thesis. But it is more complex than that: some xenoestrogens are ER agonists and other are antagonists, so their interactions with biological systems might involve the addition of negative and positive effects. One thing is certain, there is well-founded suspicion that xenoestrogens are affecting human health and wellbeing in a way unthinkable just a few decades ago.

I will attempt to bring this thinking together in this overall discussion, with a view to elucidating the bigger picture approach to the effects of xenoestrogens in humans.

8.2 Human exposures to xenoestrogens

The endocrine system is one of the body's major homeostatic control systems whose aim is to maintain normal functions and development in the face of a constantly changing environment. Like all homeostatic control systems, the capacity to maintain physiological parameters within normal bounds is finite, and when this capacity is exceeded by chemical exposures (e.g. xenoestrogens), adverse consequences can ensue (Marty*, et al.*, 2011). It is widely known that humans are continually exposed to xenoestrogens via daily food and lifestyle habits (Olea*, et al.*, 1999, Singleton*, et al.*, 2003, Thomson, 2005) which 'attack' the endocrine system by various mechanisms (see Fig. 4.2). Whilst recognising that there are various mechanisms by which environmental compounds might influence the level and balance of sex hormones in the body, this thesis focuses on dietary (e.g. natural and synthetic) and personal care product (e.g. shampoo) xenoestrogens that are known to bind and activate ER via the classical genomic pathway of ligand-dependent activation of the ERs (α and β) (Gruber*, et al.*, 2002). Many compounds with *in vitro* estrogenic activity have been detected in a wide range of foods and personal care products. Hence all humans will be exposed to xenoestrogens on a daily basis. Naturally occurring xenoestrogens include isoflavones (e.g. genistein and daidzein) and the metabolite of daidzein, equol, curcuminoids (e.g. curcumin and tetrahydrocurcumin), and the flavonoids (e.g. kaempferol). Exogenous estrogens (e.g. natural estrogens found in foods) have also been shown to have estrogenic activity including E2, estrone and estriol from dairy products, meat etc. Synthetic compounds that have been shown to have estrogenic activity and have been found in food and water include industrial chemicals (e.g. BPA), medications (e.g. EE2) and personal care products (e.g. methylparaben, butylparaben and benzylparaben) (Caldwell*, et al.*, 2010).
Xenoestrogens are everywhere. In addition, public perception may present a bias that synthetic chemicals are more risky than natural chemicals; the risk of the synthetic xenoestrogens can only be determined in the context of the total risk of all xenoestrogens, including naturally occurring phytoestrogens.
In order to understand the magnitude of the human health implications from xenoestrogen exposures, we need to know what people are exposed to. Drawing extensively on pre-existing data, the risk assessment in this thesis illustrates the extent of xenoestrogen exposures with significant inter-individual variability. However, it has also created new knowledge of data

gaps, which suggests a more comprehensive ranking of contributing xenoestrogens and an estimate of the total level of risk to estrogenic compounds from daily food and lifestyle habits is required– this is no small task.

The additive approach taken in this study suggests that combined xenoestrogens from food and lifestyle habits could have a significant biological effects, especially when compared with the endogenous levels of E2; interestingly, exposures calculated from the Canterbury women's study reported here suggest xenoestrogen exposure could potentially triple the circulating levels of estrogenicity for post-menopausal women and pre-pubertal aged girls (see Table 7.29) from their daily food and lifestyle choices (Fig. 8.1). The potential tripling of the baseline circulating estrogenicity value could mean that, in the context of breast cancer cell proliferation, there is three times as much proliferative power. Compared with normal circulating estrogen levels this increase in proliferative power is significant and could have a pharmacological effect. However, as demonstrated in Chapters 4 and 5, some calculated estrogenic loads may have anti-proliferative activity; this suggests that the concept of simple additivity does not account for all the possible exposure scenarios.

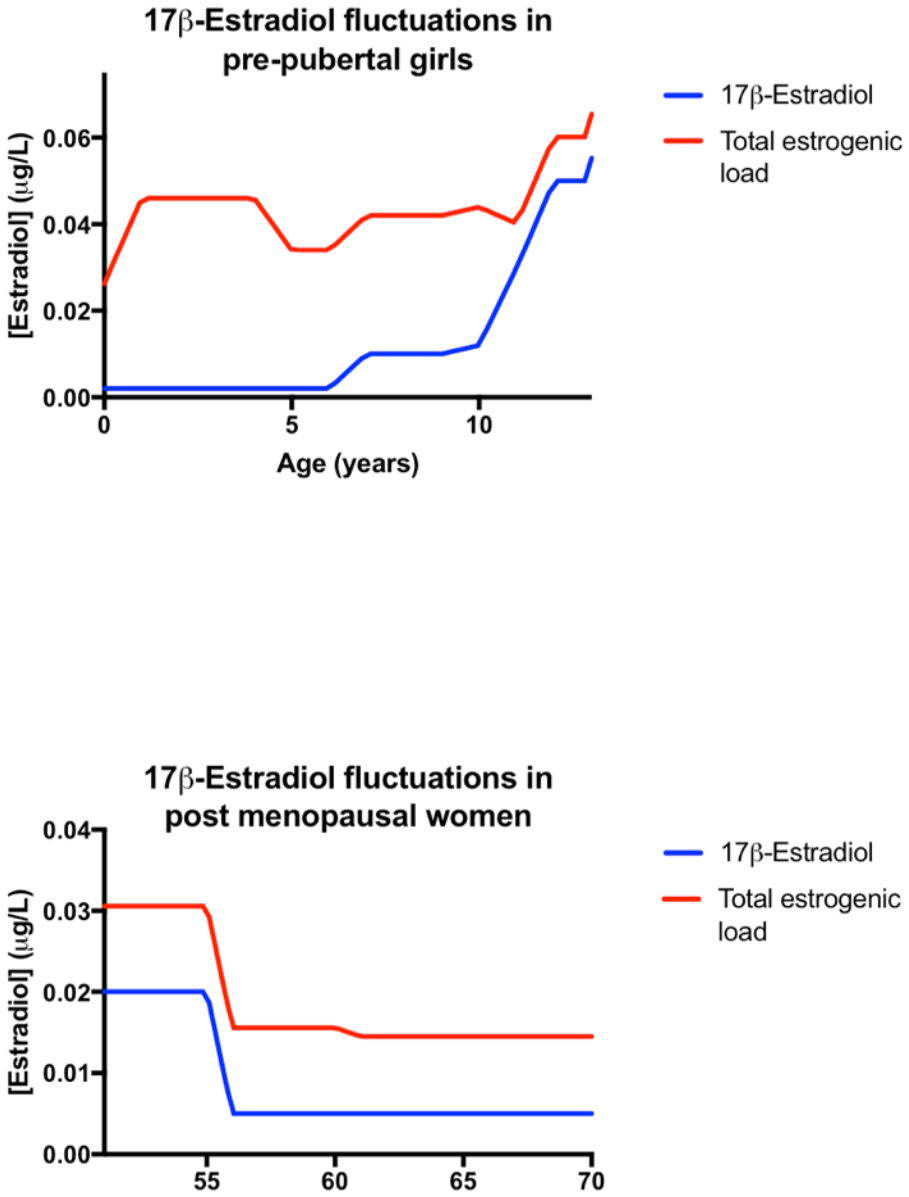


Figure 8.1: The impact of calculated xenoestrogens on the total estrogenic loads of pre-pubertal girls (top) and post-menopausal women (bottom). This clearly illustrates the significant increase in total estrogenicity by xenoestrogen exposures on these populations – this is based on data presented in Chapter 7 and the literature (Cummings*, et al.*, 1998, Elmlinger*, et al.*, 2002).

On the other hand, for child bearing age women it is hard to see how an extra 16.9% of estrogenicity from food and lifestyle habits could have an effect because a woman's body is physiologically adapted to major cyclic fluctuations of E2 levels during the estrus cycle (Fig. 8.2). Thus, the contribution from food and lifestyle habits is likely to be small in comparison to the level of naturally circulating E2.

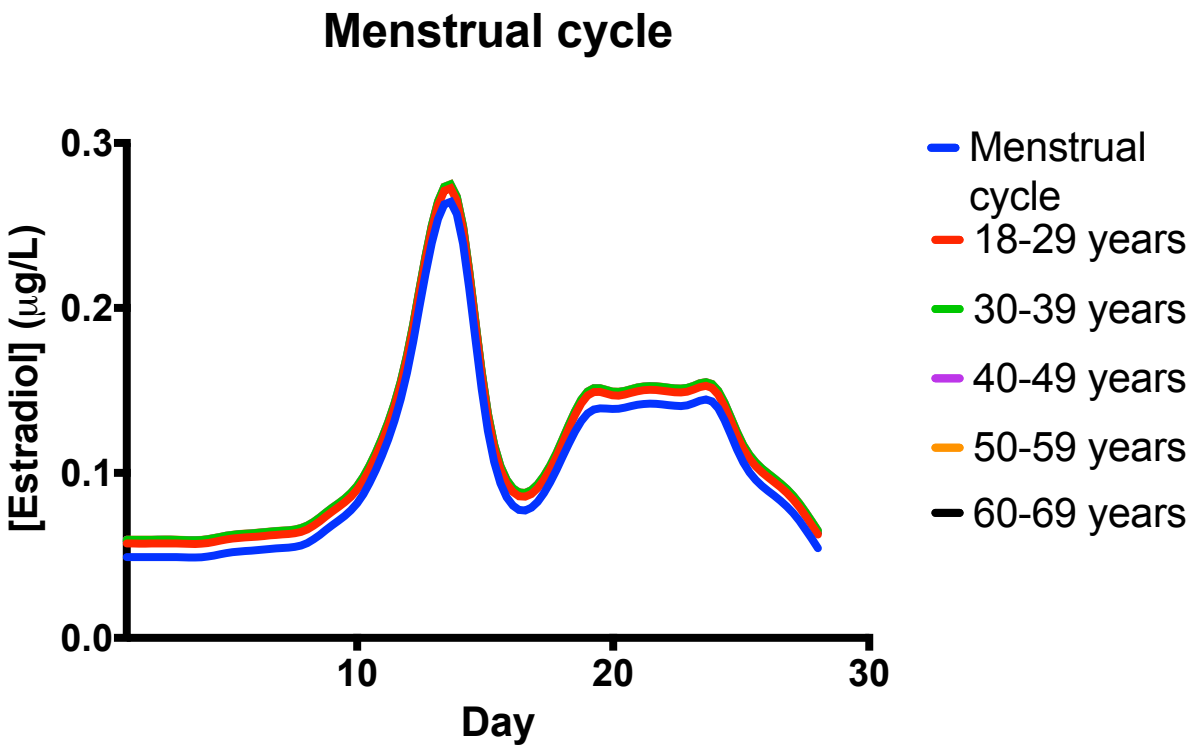


Figure 8.2: The impact of calculated xenoestrogens on the total estrogenic loads of child bearing age women determined from the average E2 levels in a menstrual cycle (blue) (data from http://commons.wikimedia.org/wiki/File:Estradiol_during_menstrual_cycle/png) and data calculated from 18-29 (red), 30-39 (green), 40-49 (purple), 50-59 (orange) and 60-69 (black) subgroups. This clearly illustrates that small influence of xenoestrogen exposure on fluctuating E2 levels during the estrus cycle.

The Canterbury women's study reported in this thesis represents the diet and life style xenoestrogen exposures that Canterbury women would be exposed to on a daily basis. They would not be expected to be markedly dissimilar from countries such as the U.S.A and Britain so perhaps these data could be extrapolated to these populations; however, it certainly would not be similar to people in Africa and most of Asia. As discussed, the diet consists of multiple and variable residues over this constitutes a cocktail effect. It's is the total estrogenic intake that causes the biological effect

There is previously published evidence of additivity of effect of low doses of xenoestrogens based on experiments using the yeast assay (Payne*, et al.*, 2000, Rajapakse*, et al.*, 2002, Silva*, et al.*, 2002) cell proliferation (Rajapakse*, et al.*, 2004) and gene expression assays (Gaido*, et al.*, 2003). However, additivity of xenoestrogen cocktails and the overall

estrogenic effects are almost impossible to predict because some xenoestrogens (e.g. genistein) have different individual estrogenic effects compared to when they are components of mixtures (Gaido*, et al.*, 2003, Rajapakse*, et al.*, 2004). In addition, this estrogenicity may vary in different target cells depending on ER isoform expression (Gaido*, et al.*, 2003). Indeed, it is clear from the results presented in this thesis that the concept of simple additivity is not necessarily the case. For example, the phytoestrogens often had opposing effects as seen in CALUX® (Chapter 5) and MCF-7 studies (Chapter 4). While modelling studies (Chapter 6) support binding of xenoestrogens to the LBC, a major finding from my work it is clear that they are also able to interact at the AF-2 site. Xenoestrogens have not previously been shown to bind to this site, which presents a more complex interaction with the ERs and greater uncertainty relating to the overall effect of exposures to xenoestrogen cocktails.

The results presented in Chapters 4 and 5 do not take into account the complexity of the potential exposure mixtures reported in the questionnaire and blood analysis studies, thus, comparisons between the individual xenoestrogen exposures might shed light on possible cocktail effects. Tables 8.1 and 8.2 shows the daily individual xenoestrogen exposures for each subgroup of women (Group 1) and pre-pubertal girls in the present study. Table 8.3 shows the concentrations in MCF-7 studies where an anti-proliferative or proliferative effect was observed. Concentrations where a negative effect in the ERα and ERβ CALUX® assays was seen are shown in Table 8.4. It is clear from these tables that the xenoestrogen exposure concentrations used in MCF-7 and CALUX® studies were of the same order of magnitude as the exposures reported from the questionnaire study; therefore, the responses from the MCF-7 and CALUX® studies are likely to be biologically relevant (because they are based on intact living cells) and thus, predict the *in vivo* situation. Furthermore, while the overall estrogenic load might have a significant biological effect in some subgroups of women and pre-pubertal girls, the exposure concentrations to the individual xenoestrogens could lead to quite different effects *in vivo*. For example, some exposure concentrations would be sufficient to reflect the anti-proliferative effects reported in MCF-7 and CALUX® studies (Chapters 4 and 5), while others would reflect the proliferative effects. Whilst some xenoestrogen exposures reported from the questionnaire study suggest an anti-proliferative biological effect, the assumptions employed (e.g. 100% absorption) when calculating the daily exposures might overestimate the total exposure concentration. This overestimation would suggest that individual xenoestrogen exposures would not be sufficient to prevent proliferation. This will be set in context later in this chapter.

Table 8.1: Calculated individual xenoestrogen exposures for the 5 subgroups of women studied.

	18-29 (M)	30-39 (M)	40-49 (M)	50-59 (M)	60-69 (M)
Genistein	4.5×10^{-9}	1.3×10^{-7}	1.26×10^{-7}	1.26×10^{-7}	2.8×10^{-8}
Estradiol	3.3×10^{-11}	1.8×10^{-11}	3.2×10^{-11}	3.2×10^{-11}	3.6×10^{-11}
Estriol	1.9×10^{-11}	1.3×10^{-11}	1.3×10^{-11}	1.3×10^{-11}	1.3×10^{-11}
Butylparaben	9.5×10^{-6}	9.6×10^{-6}	8.9×10^{-6}	9.7×10^{-6}	7.3×10^{-6}
BPA	1.2×10^{-8}	1.2×10^{-8}	1.2×10^{-8}	1.2×10^{-8}	2.9×10^{-9}
EE2	6.7×10^{-16}	6.7×10^{-16}	3.4×10^{-16}	3.4×10^{-16}	2.5×10^{-15}

Table 8.2: Calculated individual xenoestrogen exposures for the 4 subgroups of pre-pubertal girls studied.

	0-12 months (M)	1-3 years (M)	5-6 years (M)	11-14 years (M)
Genistein	6.3×10^{-10}	9.8×10^{-7}	7.9×10^{-7}	1.3×10^{-7}
Estradiol	1.4×10^{-11}	9.2×10^{-11}	1.0×10^{-10}	4.0×10^{-11}
Estriol	1.1×10^{-11}	4.5×10^{-11}	3.6×10^{-11}	2.4×10^{-11}
Butylparaben	3.9×10^{-9}	3.0×10^{-8}	3.4×10^{-7}	2.6×10^{-6}
BPA	1.9×10^{-10}	4.8×10^{-9}	1.0×10^{-8}	1.5×10^{-8}
EE2	1.7×10^{-15}	2.1×10^{-15}	2.1×10^{-15}	4.2×10^{-15}

Table 8.3: Maximum responses for individual xenoestrogens alone and in combination with E2 in MCF-7 studies.

MCF-7	Individual (M)	With E2(M)
Genistein	1×10^{-7}	1×10^{-7}
E2	1×10^{-11}	
BPA	1×10^{-8}	1×10^{-8}
Butylparaben	$1 \times 10^{-7.5}$	$1 \times 10^{-7.5}$
Estriol	1×10^{-9}	1×10^{-11}
EE2	1×10^{-12}	1×10^{-11}

Table 8.4: Calculated EC50 values for individual xenoestrogens calculated from data collected from the ERα and ERβ CALUX® assays.

CALUX®	ERα EC_{50} (M)	ERβ EC_{50} (M)
Genistein	1×10^{-7}	1×10^{-8}
E2	1×10^{-10}	1×10^{-10}
BPA	1×10^{-6}	1×10^{-6}
Butylparaben	1×10^{-6}	1×10^{-5}
Methylparaben	1×10^{-6}	1×10^{-5}
EE2	1×10^{-12}	1×10^{-10}

In the Canterbury women's study, the dose of genistein would not be sufficient to prevent breast cancer cell replication in the 18-29 and 60-69 years subgroups, whilst the 30-39, 40-49 and 50-59 subgroups appear to have a sufficient dose to prevent replication. Interestingly, all subgroups of women in the Canterbury women's study would be exposed to sufficient doses of butylparaben to prevent breast cancer cell proliferation. In addition, the exposure concentrations for E2, BPA, EE2, Estriol and methylparaben would all be sufficient to induce proliferative effects. This is interesting in the context of breast cancer where proliferation of transformed cells is crucial to tumour growth. On the other hand, the MCF-7 eight component combination study did take into account the complexity of the exposures reported in the Canterbury women's study. The response observed in this experiment clearly shows that the proliferative effects of BPA, E2 and EE2 are ameliorated by the anti-proliferative effects of genistein, kaempferol, tetrahydrocurcumin, butylparaben and estriol. Therefore, the cocktail effect of the calculated xenoestrogen exposures from the Canterbury women's study is likely to be far more complex than just simple additivity. For example, the estrogenic effect of xenoestrogen exposure cocktails is related to the identity of the individual components and to their exposure concentrations. The biological interaction between xenoestrogens is complex– this makes predictions of cocktail effects very difficult.

In addition, the use of hormone contraceptives and HRT significantly increases the overall estrogenic load that women are exposed to. The calculated xenoestrogen exposure levels in

child bearing age women appear to be a 'drop in the bucket' compared to the concentrations of estrogens in their medications. Therefore, the biological effect of these medicines could have serious implications for breast cancer risk. While the pharmaceutical goal of HRT has been achieved (i.e. minimising menopausal symptoms), on the other hand there is the potential for significant cell-based side effects (e.g. increased breast cancer cell proliferation). Indeed, an increase in breast cancer incidence due to the use of hormone replacement therapies is well documented (Rossouw, *et al.*, 2002). As a result of this, the use of HRT has significantly decreased over the last decade (Antoine, *et al.*, 2014, Canfell, *et al.*, 2008, Katalinic, *et al.*, 2009, Lambe, *et al.*, 2010, Park, *et al.*, 2012, Salagame, *et al.*, 2016, Zbuk, *et al.*, 2012). Therefore, it is surprising to find that 5.0% of the women in the Canterbury study had been prescribed HRT. On the other hand, a recent Danish study also found that the risk of breast cancer was higher among women who were currently taking, or had recently used hormonal contraceptives compared to women who had never used them (Morch, *et al.*, 2017). While Morch *et al.*, (2017) found that the risk increased with longer duration of use, the overall risk increase was small (i.e. <5%). When assessing overall estrogenic exposures in women prescribed hormone contraceptives it is easy to understand why they might increase breast cancer risk. This likely applies to all other xenoestrogens; however, the calculated circulating levels of EE2 and E2 in estrogen medications are much higher that endogenous circulating E2 concentrations, therefore, the high doses from the medications could outweigh the protective effects of some xenoestrogens.

8.3. Understanding the Xenoestrogen Exposure Scenario

It is clear from the Canterbury women's exposure study that each woman or pre-pubertal daughter is exposed to a complex array of xenoestrogens in their day-to-day lives. To add to this complexity, their 'natural' circulating E2 levels can vary significantly (Cummings, *et al.*, 1998, Elmlinger, *et al.*, 2002) depending on the developmental stage, making predicting the effects of xenoestrogens exposures very difficult. While a battery of validated assays, both *in vivo* and high-throughput *in vitro*, have been developed to screen for agonistic and antagonistic xenoestrogens, the cost of comprehensively testing the multitude of synthetic and natural xenoestrogens to which women might be exposed would be formidable (Falconer, *et al.*, 2006), let alone the myriad potential exposure combinations. Over the last decade, barely the tip of the iceberg of the potentially estrogenic chemical universe has been tested – this would be almost impossible (Birnbaum, 2013, Falconer, *et al.*, 2006). Thus, suffice to say that a full xenoestrogen assessment across the plethora of daily chemicals exposures is a

daunting prospect. Therefore, computational techniques are becoming increasingly important to complement experimental studies. Indeed, I have used *in* silico modelling in this thesis to study complex interactions of xenoestrogen mixtures at ER binding sites with a view to predicting biological effects.

In this thesis, rapid *in silico* screening was used not only to help identify and prioritise which xenoestrogens to study, but also to reduce the number of compounds tested. While a number of studies have reported models to screen for potential ligands based on docking alone, there is still uncertainty whether docking studies adequately represent the *in vivo* situation (Kitchen*, et al.*, 2004, Zhang*, et al.*, 2013). Therefore, in order to validate the docking experiments carried out in this thesis, correlations between theoretical and experimental data were determined. For example, correlations between calculated RRD and IFD binding energies with the corresponding EC_{50} values from ERα and ERβ CALUX® assays were determined for selected ligands (Chapter 5) (Fig. 8.3). The R^2 and P values show a non-linear correlation between computational docking and CALUX® data sets. Therefore, RRD and IFD are not good predictors of CALUX® results and vice versa. As discussed in Chapter 5, some CALUX® data exhibited supramaximal responses, both for individual xenoestrogens and for combinations which could explain the correlations. The increase in the maximum response in the CALUX® assay (i.e. supramaximal response) will shift the EC_{50} value to the right (Fig. 8.4) which will give artefactually low EC_{50} values. Thus, this might explain the poor correlations between the calculated RRD and IFD binding energies and the corresponding CALUX® EC_{50} values.

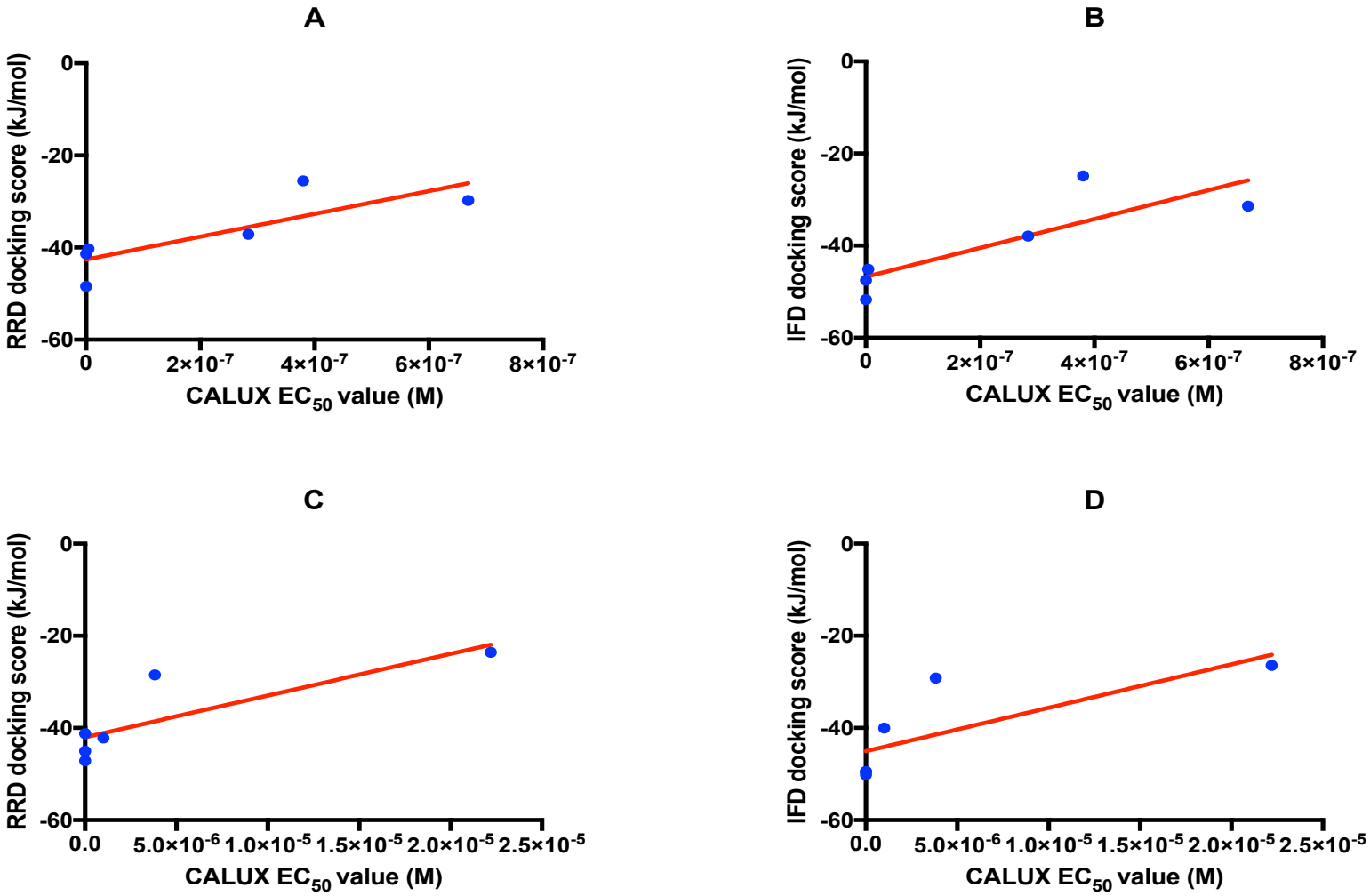


Figure 8.3: Correlations for calculated docking scores and CALUX® EC_{50} values. A=ERα correlation between RRD docking scores and CALUX® EC_{50} values; B = ERα correlation between IFD docking scores and CALUX® EC_{50} values. C = ERβ correlation between RRD docking scores and CALUX® EC_{50} values. D= ERβ correlation between IFD docking scores and CALUX® EC_{50} values.

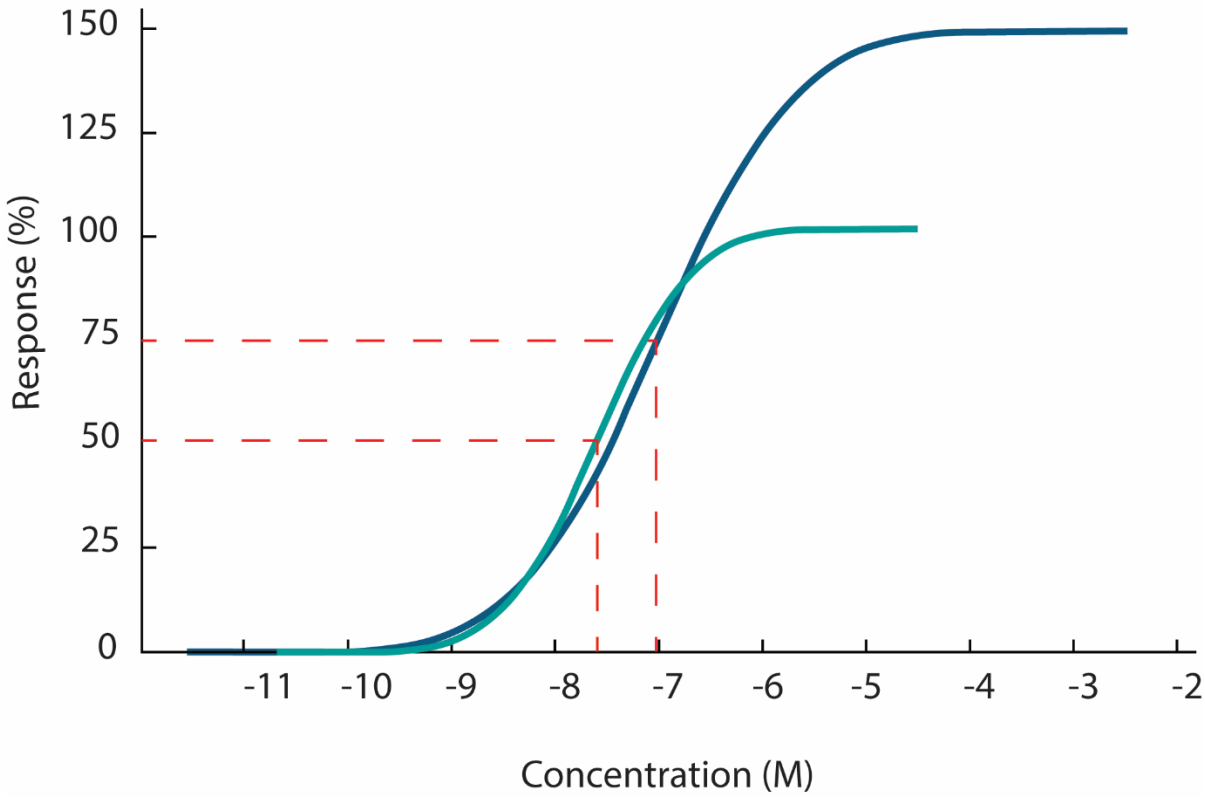


Figure 8.4: Illustration of supramaximal effects on calculated EC_{50} values in the ERα and ERβ CALUX® assays.

8.4. The Intimate Relationship between Xenoestrogens and ERs

Decades of research has revealed extraordinary insights into how ERs function - a immensely complex picture is emerging. Historically, ERs were thought to be standalone transcription factors acting solely through genomic mechanisms that impact on nuclear ER function; however, recent research has shown ER activity requires the highly co-ordinated accumulation of dozens of coregulatory proteins that perform a multitude of functions (e.g. the ability to 'open' chromatin, making the previously compacted DNA assessible to transcriptional machinery) (Zhou, *et al.*, 2014). In addition, intracellular concentrations of ERs result in a dynamic balance between ER synthesis and ER breakdown/degradation (Glass, *et al.*, 2000, Green, *et al.*, 2007). This process requires altered phosphorylation status of ERs and the interaction of ERs with several proteins including ubiquitin ligases and ubiquitin binding proteins. To add to the complexity, cross-talk between ERs and activated growth factor receptors and their downstream kinases (e.g. non-genomic signalling) such as the HER2 mediated MAPK pathway, has been shown to play a major role in activating ERs. This highly coordinated interplay between genomic and non-genomic

signalling pathways plays a crucial role in the processes of cell physiology and highlights the role of the intimate intracellular relationship of ER mechanisms of action.

The highly coordinated interplay between genomic and non-genomic pathways is exploited by xenoestrogens which then exert their estrogenic effects. However, xenoestrogens also exhibit concentration-dependent mixed agonist/antagonist effects suggesting an even more complex interaction with ERs. Low exposure concentrations of some xenoestrogens (e.g. genistein, kaempferol) result in a stimulatory (agonist) effect on cell proliferation; however, higher exposure concentrations might result in the conversion from agonist to antagonist activity (e.g. inhibition of cell proliferation). The two-site binding model could explain this activity, whereby a primary, high affinity binding site (e.g. LBC) is responsible for the agonist activity and a secondary, low affinity binding site (e.g. AF-2) is responsible for the antagonist activity. This model has been proposed previously to explain the mixed agonist/antagonist response of the breast cancer drug tamoxifen with studies showing a unified two-site model to account for the phenomenon (Jensen*, et al.*, 2004, Kojetin*, et al.*, 2008, Wang*, et al.*, 2006). Indeed, evidence from *in silico* studies reported here (Chapter 6) corroborate the two-site binding model which is evidenced by high calculated binding energies for LBC interactions (e.g. the primary, high affinity site) and low calculated binding energies for AF-2 interactions (e.g. the secondary, low affinity site). Therefore, high ligand concentration is required to saturate the LBC and allow spill-over to AF-2 and initiate conformational change. Thus, with multiple ligands and interacting two binding sites to consider, predicting the overall cocktail effect is now even more complex. For example, one would need to consider at least three different individual binding equilibria from a two component xenoestrogen mixture of E2 and genistein in order to begin to predict the biological outcomes of dual interactions with ERs: 1) the individual ligand binding equilibria for the LBC; 2) the individual binding equilibrium for AF-2; and 3) the individual binding equilibrium between LBC and AF-2 (Fig. 4.9). Thus, because E2 binding to the LBC is favoured relative to genistein, E2 will outcompete genistein for LBC occupancy; this will increase the free genistein concentration, thus, potentially facilitating genistein spill over to AF-2. If genistein alone was present in the LBD, since it has a lower LBC binding affinity, a higher genistein exposure concentration would be necessary to result in AF-2 spill-over. Therefore, the higher the LBC ligand binding affinity, the lower the ligand

concentration that is required for AF-2 spill over; however, spill over is also dependent on AF-2 ligand binding affinities because the higher the AF-2 binding affinity the lower the concentration of free xenoestrogens required to bind to the AF-2 site. This is exemplified by experiments in MCF-7 cells and the ERα and ERβ CALUX® assay reported in this thesis in which negative modulatory effects were observed on proliferation and luciferase response. Therefore, for a better understanding of xenoestrogen cocktail effects, the ratio between AF-2/LBC binding energies is crucial.

The LBC/AF-2 binding affinity ratios were determined for ERα from *in silico* studies and compared with the maximum proliferative responses elicited by xenoestrogen mixtures in the MCF-7 studies. Excitingly, this illustrates a clear relationship between binding energy ratios and MCF-7 cell proliferation. The P value is 0.0008 suggesting a statistically significant correlation between the data sets (Fig. 8.5). This supports the hypothesis that LBC/AF-2 interplay is a key facet of the biological outcomes of exposures to xenoestrogen cocktails. The interactions and interplay between binding sites could have significant biological implications and be important in drug development approaches involving designing small molecules to target AF-2 directly (i.e. circumventing the need to saturate the LBC).

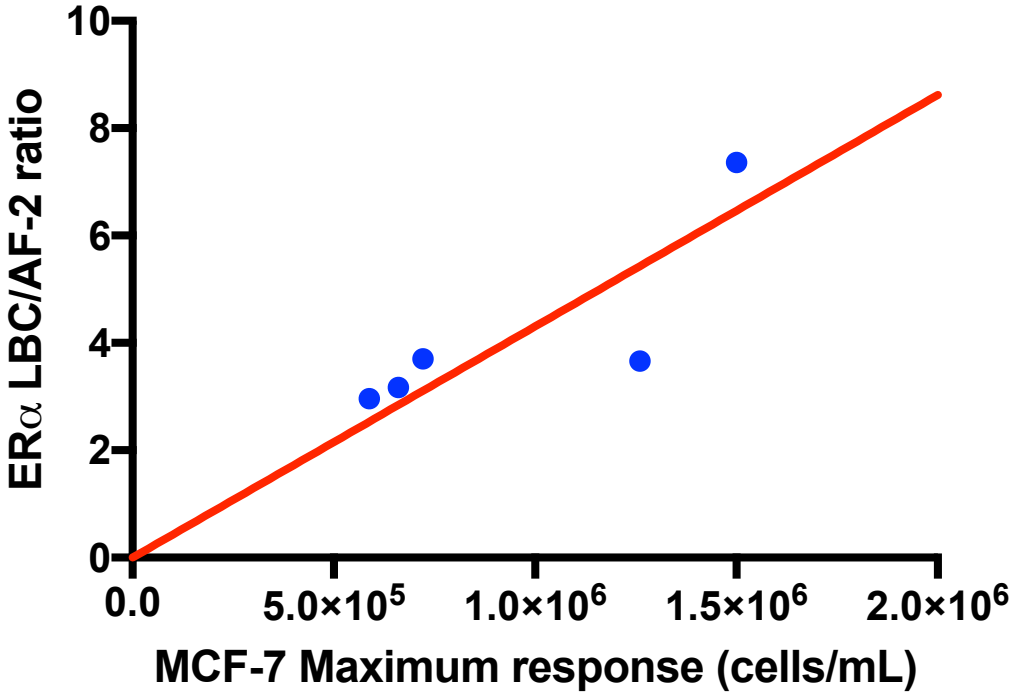


Figure 8.5: Correlation for the calculated LBC/AF-2 ratio and the maximum proliferative response in MCF-7 studies.

8.5. Biological Consequences of Xenoestrogen/AF-2 Interactions

The implications of xenoestrogen interactions at AF-2 could have significant impact on ER-mediated genomic and non-genomic mechanisms in the breast. In ER+ve breast cancer the presence of E2, and subsequently exposure to xenoestrogens, provides 'permission' for the cell to proliferate (Goodsell, 2002); however, at high concentrations some xenoestrogens (e.g. genistein) could rescind this permission by augmenting the reception of the message, e.g. via its interaction with AF-2. These AF-2 interactions could have numerous downstream effects on estrogen-related biological outcomes which can impact the way breast cancer cells functions (Fig. 8.6). For example, in an ER+ve breast cancer cell, ERα is highly expressed while ERβ has a significantly lower level of expression (Lim*, et al.*, 2016). Increasing the ERα/ERβ ratio amplifies ERα–mediated cellular signalling (e.g. cell cycle progression); however, if a xenoestrogen interacts with the ERα AF-2 site it might block the reception of this signal, rendering it invalid. This would result in an inhibitory effect on ERα-mediated cellular functions, ultimately leading to overall anti-proliferative

effects. Indeed, this was observed in the MCF-7 cell studies reported in this thesis where some xenoestrogens showed concentration-dependent anti-proliferative effects. There are a number of explanations for the anti-proliferative effects induced by xenoestrogens, including crosstalk between genomic and non-genomic signalling pathways, ERβ antagonism of ERα-mediated responses, and recruitment and availability of coregulatory proteins. However, the anti-proliferative effects could also be a result of LBC/AF-2 interplay. Indeed, the general consensus of opinion re the mechanism of mixed agonist/antagonist effects is that it is a result of ERβ preferential binding (Lecomte *et al.,* 2017) and its subsequent modulatory effect on ERα. Interestingly, the MCF-7 and ERα CALUX® xenoestrogen exposure studies reported here both illustrate the modulatory effects of some xenoestrogens; however, ERβ is not expressed in the ERα CALUX® cell line. This suggests that the modulatory effects by some xenoestrogens in both studies is not a result of ERβ control of ERα and eliminates the possibility of ERβ modulating ERα-mediated actions in MCF-7 cells. It also highlights the importance of ERα and ERβ ratios in breast cancer. Indeed, it is well documented that the ERα/ERβ ratio in breast cancer is much higher than in normal breast tissue due to dysregulation of ERα and lower expression of ERβ Kim *et al*., 2012; Leung *et al*., 2012; Roger *et al.,* 2001). Therefore, AF-2 interactions could downregulate ERα-mediated cell signalling, resulting in an apparently lower ERα/ERβ ratio. The apparent lowering of the ERα/ERβ ratio due to interactions with AF-2 could restore apparently 'normal' function to the breast cancer cell; e.g. ERβ-mediated control of ERα cell signalling.

On the other hand, it has also been suggested that the recruitment and availability of co-regulatory proteins might provide an explanation for the unique mixed agonist/antagonist effects of some xenoestrogens. While it has been shown that xenoestrogens do recruit different co-activators (Kraichely*, et al.*, 2000, Nishikawa*, et al.*, 1999, Paige*, et al.*, 1999, Parker*, et al.*, 2000, Routledge*, et al.*, 2000, Wong*, et al.*, 2001), the mechanism of interference is likely more complex, with xenoestrogens blocking the assembly of coregulatory protein complexes on the DNA rather than differential recruitment alone. Interfering with co-regulatory protein assembly could affect transcription and epigenetic mechanisms elicited by ERs resulting in the same

effects observed in MCF-7 and CALUX® studies. This could also be a result of AF-2 interactions restoring the balance between ERα and ERβ mediated responses.

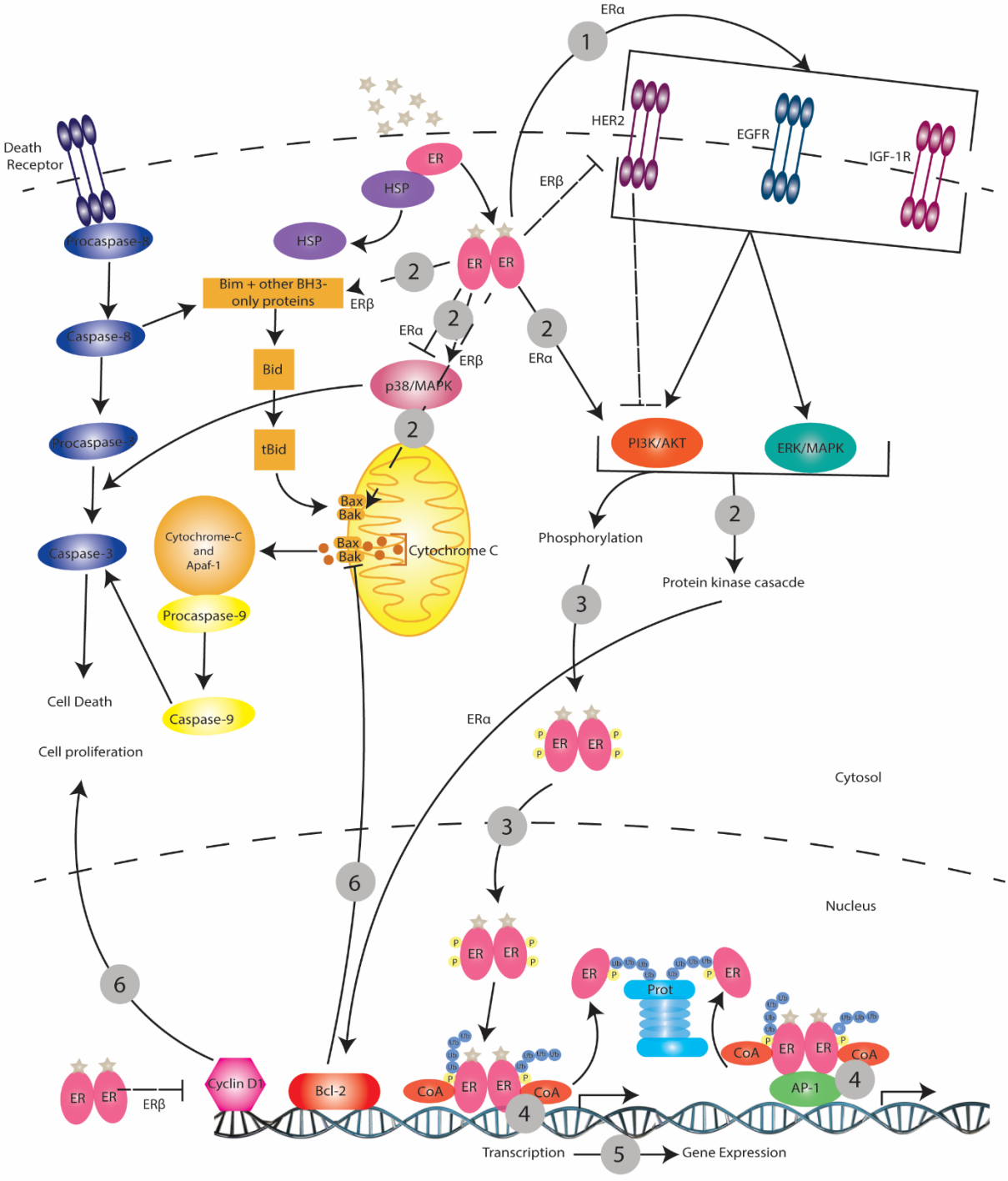


Figure 8.6: Different ways in which xenoestrogens might alter ER mechanisms of action via AF-2 site binding. 1) Modulation of growth factor receptors that ultimately activate gene transcription that lead to regulation of apoptosis and the cell cycle. 2) Modulating cell signalling pathways that can be activated by cytoplasmic ERs, ultimately leading to changes in the regulation of apoptosis and the cell cycle. 3) Modulating cell signalling pathways that result in phosphorylation of ERs. 4) Disruption of co-regulatory protein complex formation, inducing alterations of epigenetic signals and/or ER degradation. 5) Epigenetic alterations in DNA and histone proteins that induce alterations in ER-mediated transcription. 6) Inhibiting/stimulating transcription of genes regulating apoptosis and the cell cycle. (Elements of this diagram were derived from (Spencer, 2016, Zhou*, et al.*, 2014). The concept of the entire diagram is the authors)

While this is the first report that the mechanism of a possible two-site model is used to explain such effects, a number of studies have reported non-genomic signalling pathways changes that would be expected by such interaction. For example, high concentrations of genistein were found to increase the phosphorylation of p38 (Shim, *et al.*, 2007), increase the Bax/Bcl-2 ratio (Prietsch, *et al.*, 2014), cause a G2/M phase cell cycle arrest so decreasing the duration of the proliferative S-phase (Chen, *et al.*, 2003), and inhibit the activation of the IGR-1R/AKT and MAPK signalling pathways (Uifalean, *et al.*, 2016). Interestingly, Uifalean *et al.,* (2015) reported that low exposure levels of genistein in MCF-7 culture experiments resulted in a prolfierative effect, akin to E2, by activating IGR-1R/AKT and MAPK signalling pathways. Similar effects have been found in cultured MCF-7 cells exposed to curcumin and tetrahydrocurcumin where an increase in Bax/Bcl-2 ratio and cell cycle arrest at the G0/G1 phase was observed (Han, *et al.*, 2016, Lv, *et al.*, 2014, Patel, *et al.*, 2015). Published studies measured biological endpoints (e.g. Bax/Bcl-2) and, therefore, do not necessarily explain the mechanism of the underlying gene regulatory changes. AF-2 interactions may well be the pivotal to understanding the role of ER in these cell-signalling pathways because of the key role that AF-2 plays in ER-mediated biological control.

8.6. How could AF-2 Ligand Interactions be Utilised to Treat Breast Cancer?

Current breast cancer drug development strategies focus on developing small molecules that bind to the ER LBC. However, with a better understanding of ligand binding characteristics and the role of AF-2, perhaps in the future, AF-2 targeted drugs are likely to be important. Indeed tamoxifen, which was designed to bind to the LBC, also binds to the AF-2 site (Wang, *et al.*, 2006). For many years tamoxifen, and other type I anti-estrogens (e.g. raloxifene), were noted to exhibit unusual concentration-dependent mixed agonist/antagonist activity in cultured breast cancer cells (e.g. MCF-7). These studies and others led to the proposal of a two-site binding model to account for this unusual activity (Jensen, *et al.*, 2004), where the occupancy of the LBC relates to agonist activity and occupancy of the AF-2 relates to antagonist activity (Kojetin, *et al.*, 2008). The same mixed agonist/antagonist effects were seen in the xenoestrogen exposure studies reported here. This suggests that, like tamoxifen, some xenoestrogens elicit their antagonist effects via the AF-2 site. Therefore, with an

increased understanding of xenoestrogen/AF-2 interactions this knowledge could be utilised in drug development strategies.

The introduction and widespread use of tamoxifen in the post-menopausal population has resulted in significant improvements in the survival of women with ER+ve breast cancer (Dowsett*, et al.*, 2015). It is estimated that almost half a million women in the UK are alive today because of the use of tamoxifen in the treatment of ER+ve breast cancer (Jordan, 2003). Therefore, ER directed therapies represent an important and successful cornerstone strategy in the management of ER+ve breast cancer (Lim*, et al.*, 2016). However, approximately 30% of patients experience relapse due to inherent or acquired resistance to tamoxifen (Lonning, 2000). Still their increased life expectancy prior to relapse is significant.

On the other hand, multi-pronged treatments such as aromatase inhibitors to reduce E2 biosynthesis with tamoxifen which inhibits the effects of E2 at the ER have been introduced. In addition, development of the potent ER antagonist fulvestrant has led to stepwise improvements in disease control and outcomes for women with metastatic disease (Mehta*, et al.*, 2012, Robertson*, et al.*, 2012). However, breast cancer cells circumvent either ER blocking (e.g. following treatment with tamoxifen), low levels of ER activity (e.g. following treatment with fulvestrant) or low levels of E2 (following treatment with aromatase inhibitors) often leading to unresponsive metastatic breast cancer. Therefore, it is not surprising that a 3rd line of therapies has been introduced. For example, alternative target therapies have recently been used in combination with ER-directed therapies to improve survival outcomes. This represents a major advance in the treatment options for patients with ER+ve metastatic disease. These third line therapeutics include drugs that target the PI3K cell signalling pathway such as everolimus, which is an inhibitor of mTOR downstream of PI3K (Bachelot*, et al.*, 2012, Baselga*, et al.*, 2012, Piccart*, et al.*, 2014). There is no question about the efficacy of these therapies and the impact they have had on the global breast cancer burden, but perhaps we should question the dogma of developing more and better LBC antagonists augmented with biochemical means of minimising E2 levels in favour of exploring the potential of AF-2 as a therapeutic target.

8.7. Breast Cancer Prevention

The human breast harbours one of the most significant cancers in the world, affecting up to one out of eight women. An estimated 1 million people worldwide will be identified yearly and about 500,000 new and existing patients worldwide will die (WHO, 2013). Currently, the most important determinant of breast cancer treatment success is early detection. Late detection significantly reduces treatment success because of the potential of metastasis, even though a multitude of treatment regimens are available (e.g. surgery, hormone therapy). Once the disease becomes disseminated, cure is not possible; although long survivals are well documented. With the continuous global escalation in incidence, and the economical limitations of progress, there is still risk that overall mortality might increase in the future. Clearly, there is an urgent need to slow the incidence rates by affordable means.

While early detection and effective treatments have reduced the global breast cancer burden, prevention is an important strategy to minimise the burden further. Prevention relies on understanding and identifying breast cancer risk factors. The currently acknowledged breast cancer risk factors are genetics, age, lifestyle and age of parity. Perhaps we should focus on lifestyle risk as a means of further reducing burden. Lifestyle risk include diet, alcohol consumption, exercise, etc (Hiatt*, et al.*, 2018). Understanding dietary risk factors better might present a means of reducing risk.

The well documented relationship between phytoestrogen consumption and breast cancer risk has fuelled the widespread belief that some xenoestrogens can reduce breast cancer risk (Bilal*, et al.*, 2014, Gikas*, et al.*, 2005, Pelekanou*, et al.*, 2011). Results presented in this thesis clearly support this argument and suggest a concentration dependent protective effect elicited by the phytoestrogens studied, along with the anti-microbial agent butylparaben and the endogenous estrogen, estriol. The possibility of the proposed two-site binding model could provide new opportunities in preventative therapies which aim to target AF-2 site binding, exploiting the intricate ER control mechanisms. The dysregulation of ERα expression is a hallmark of breast cancer (Lim*, et al.*, 2016); thus, if xenoestrogens were able to target the ER, preventing the dysregulation of ERα and maintaining the ERα/ERβ ratio control, they could be a prime candidate for preventative drug therapies. On the other hand, due to the prevalence of xenoestrogens in food and the environment

women could simply employ lifestyle changes that might reduce their breast cancer risk. For example, increasing their vegetable consumption (many vegetables contain estrogenic flavonoids, e.g. isoflavones) and reducing the use of plastic (many plasticisers are estrogenic, e.g. BPA from polycarbonates) in their households. However, as simple as this might sound, there are many complicating factors because of the uniqueness of individuals exposure scenarios. This would mean that a risk factor-based prevention strategy would have to be personalised.

8.8. A Role for Regulation?

One of the most difficult problems related to xenoestrogens is establishing risk assessment strategies for potential adverse effects on human health. Toxicological assessments take into considerations both the biologic potency (in a negative sense, e.g. NOAEL) of a chemical as well as possible or known exposure scenarios. For xenoestrogens, there are many complicating factors with respect to differential toxicity in relation to gender, age and period of development - this is further complicated by differences in ER isoform expressions in developmental stages (Singleton*, et al.*, 2003). In addition, recently described non-genomic effects of E2 could provide mechanisms for xenoestrogen action at concentrations several orders of magnitude lower than those required for its genomic modes of action. However, cause affect relationships between xenoestrogens and breast cancer have not been established. Furthermore, it must be realised that the estrogenicity of a chemical does not necessarily equate to breast cancer development (Xu*, et al.*, 2008), which is reflected by the significant dietary consumption of phytoestrogens and their apparent beneficial effects. This poses a conundrum for regulators where classically regulation of chemicals is limited by the maximum exposure effect (i.e. NOAEL); however, in the case of phytoestrogens the inverse is true. Phytoestrogens clearly have a minimum exposure concentration which leads to the anti-proliferative effects; therefore, regulators would need to consider the minimum exposure concentration in order to determine the beneficial effects. Nevertheless, because of the presence of some xenoestrogens in food and the environment, the high potential for human exposure, and their accumulation in biological matrices, it is crucial to understand their modes of action and relative risks in a breast cancer context.

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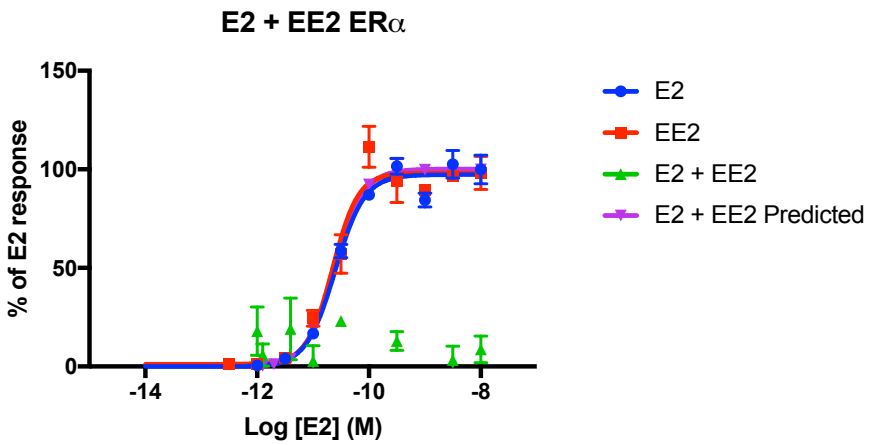
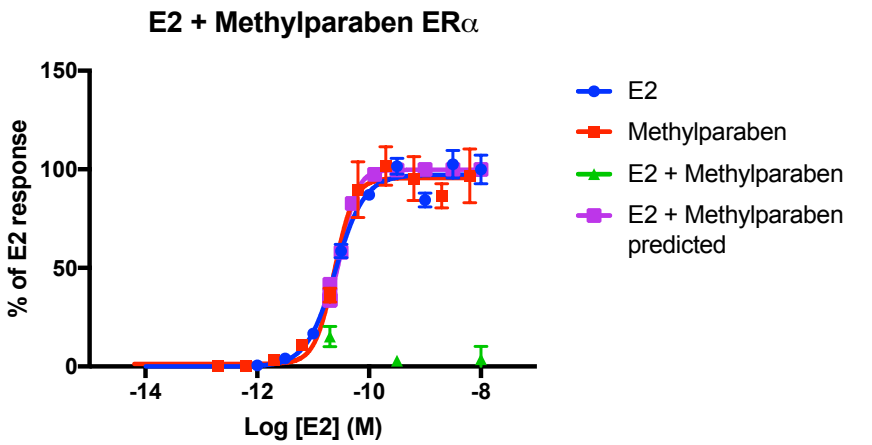
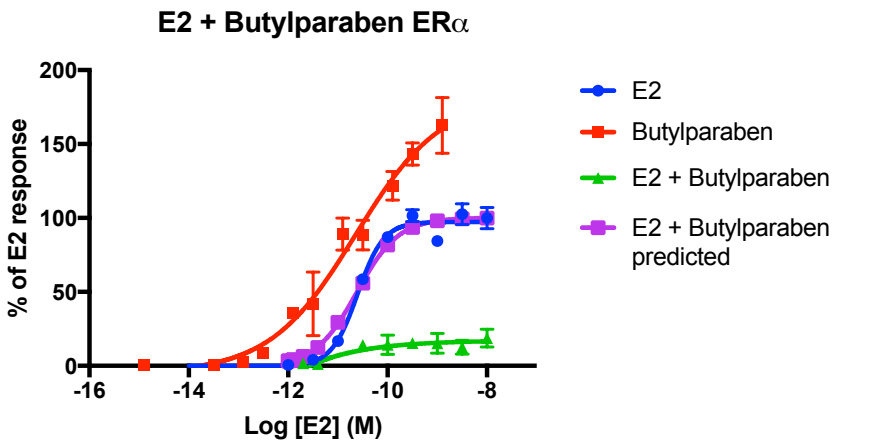
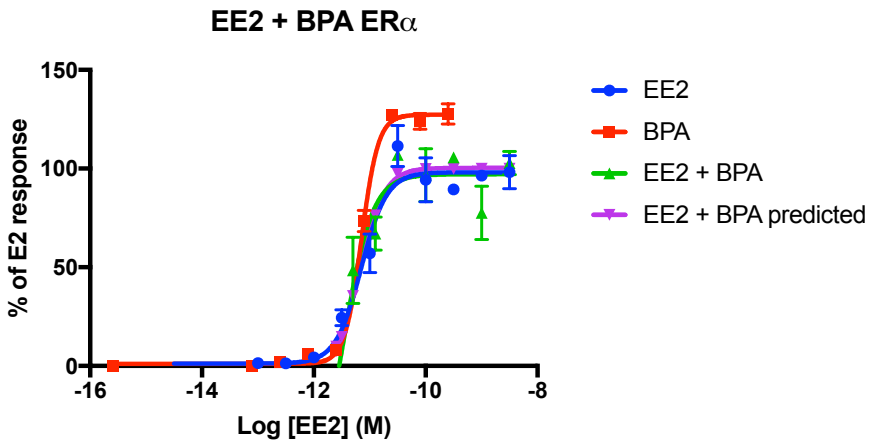
Appendices

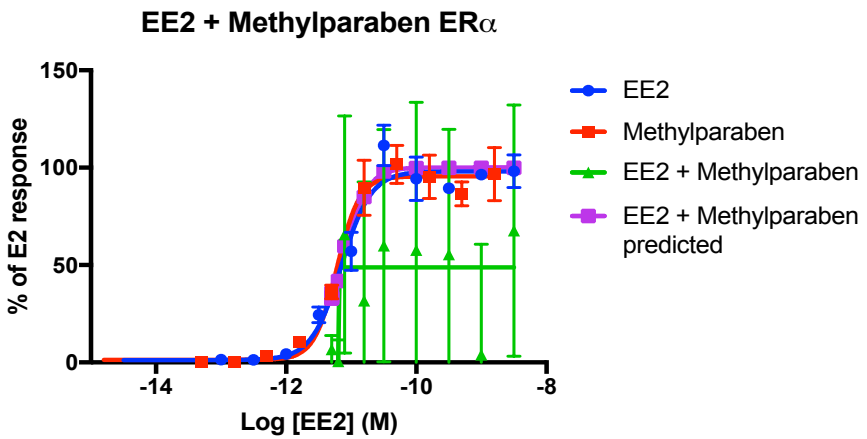
Appendix 1: Results of Individual and 2 Component Combinations of Xenoestrogens in the ERα CALUX® Assay not Included in Chapter 5. Results are Presented using Non-linear Regression Analysis with Errors Expressed as SEM.

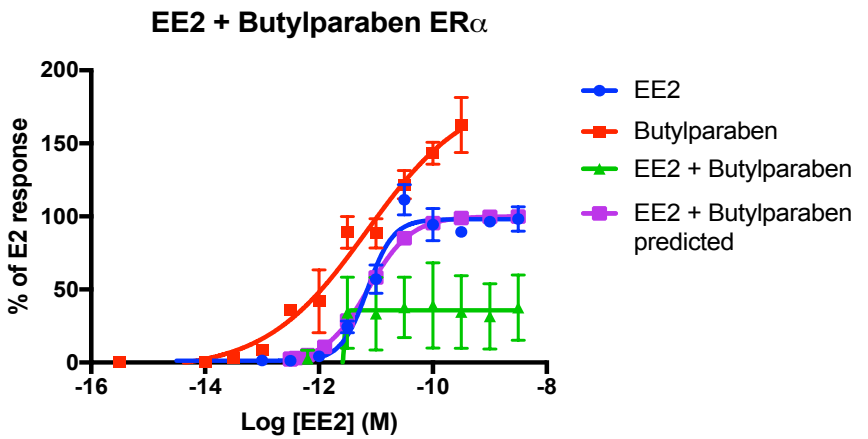

E2 + EE2 ERα
% of E2 response
150
100
50
0
-14
-12
-10
-8
Log [E2] (M)
E2
EE2
E2 + EE2
E2 + EE2 Predicted

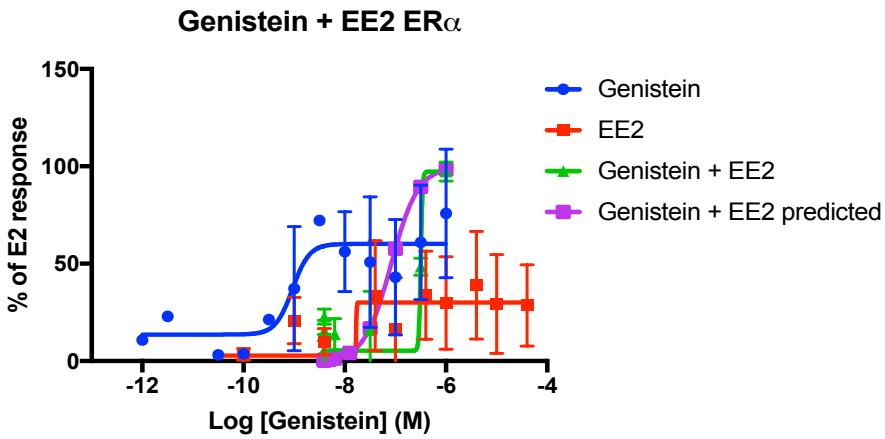

E2 + Methylparaben ERα
% of E2 response
150
100
50
0
-14
-12
-10
-8
Log [E2] (M)
E2
Methylparaben
E2 + Methylparaben
E2 + Methylparaben predicted

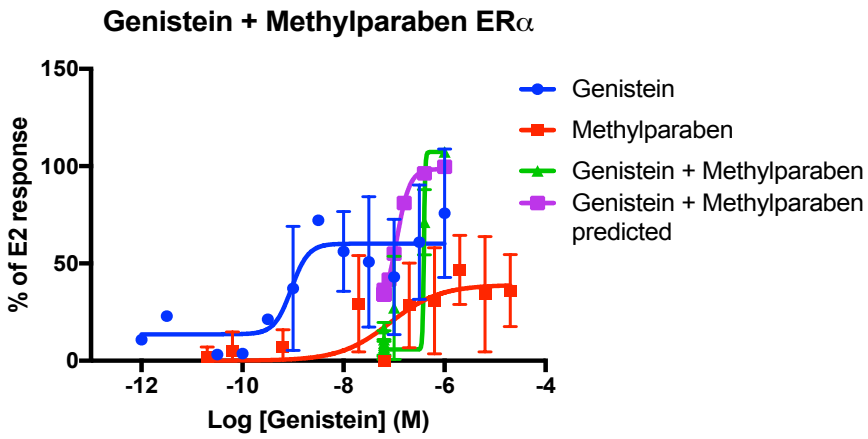

E2 + Butylparaben ERα
% of E2 response
200
150
100
50
0
-16
-14
-12
-10
-8
Log [E2] (M)
E2
Butylparaben
E2 + Butylparaben
E2 + Butylparaben predicted

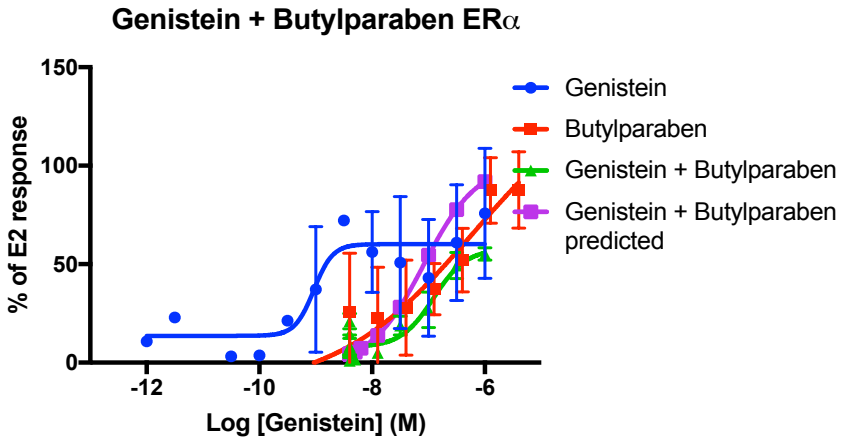

EE2 + BPA ERα
% of E2 response
150
100
50
0
-16
-14
-12
-10
-8
Log [EE2] (M)
EE2
BPA
EE2 + BPA
EE2 + BPA predicted

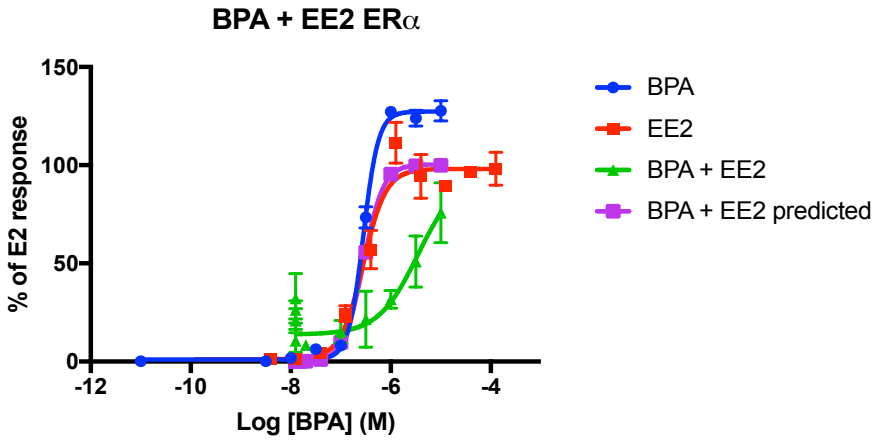

EE2 + Methylparaben ERα
% of E2 response
150
100
50
0
-14
-12
-10
-8
Log [EE2] (M)
EE2
Methylparaben
EE2 + Methylparaben
EE2 + Methylparaben predicted

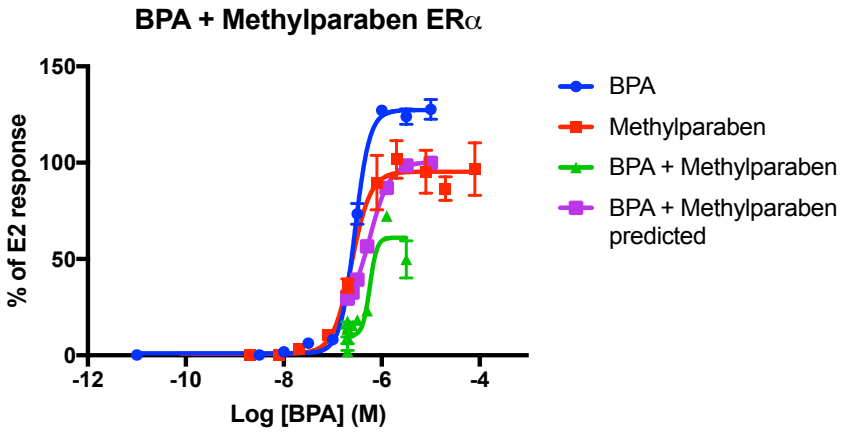

EE2 + Butylparaben ERα
% of E2 response
200
150
100
50
0
-16
-14
-12
-10
-8
Log [EE2] (M)
EE2
Butylparaben
EE2 + Butylparaben
EE2 + Butylparaben predicted

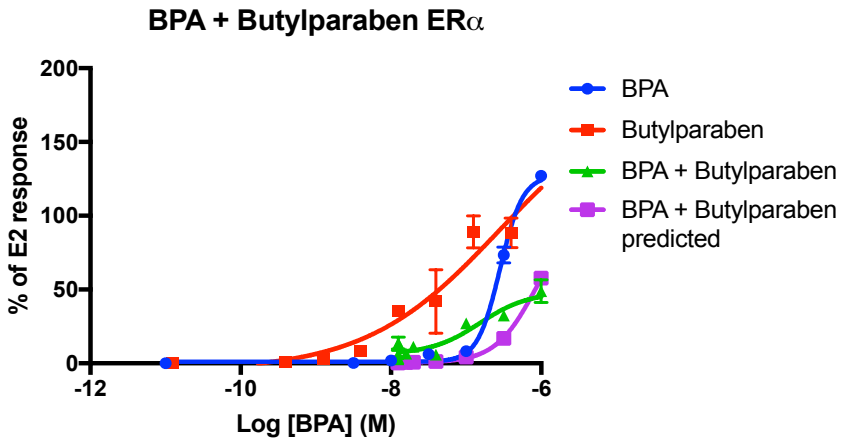

Genistein + EE2 ERα
% of E2 response
150
100
50
0
-12
-10
-8
-6
-4
Log [Genistein] (M)
Genistein
EE2
Genistein + EE2
Genistein + EE2 predicted

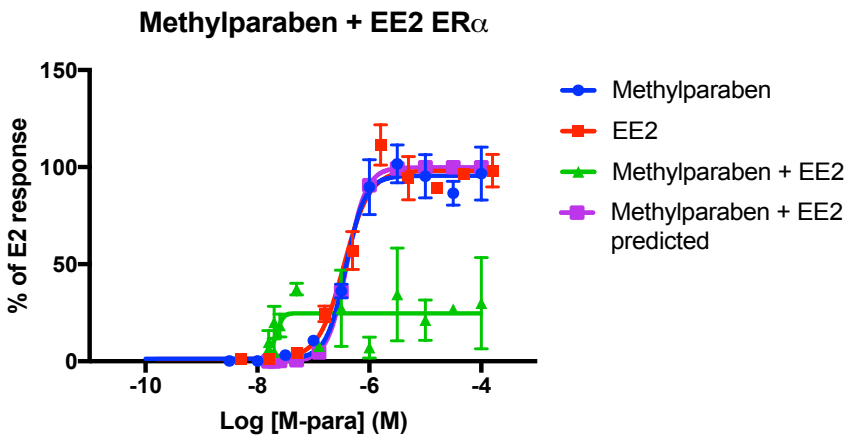

Genistein + Methylparaben ERα
% of E2 response
150
100
50
0
-12
-10
-8
-6
-4
Log [Genistein] (M)
Genistein
Methylparaben
Genistein + Methylparaben
Genistein + Methylparaben predicted

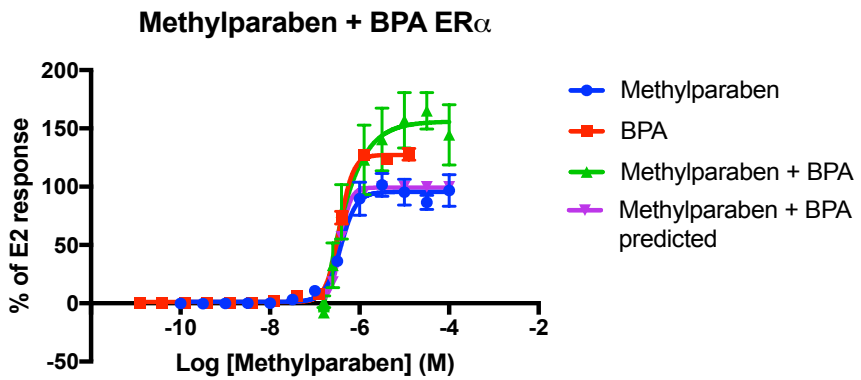

Genistein + Butylparaben ERα
% of E2 response
150
100
50
0
-12
-10
-8
-6
Log [Genistein] (M)
Genistein
Butylparaben
Genistein + Butylparaben
Genistein + Butylparaben predicted

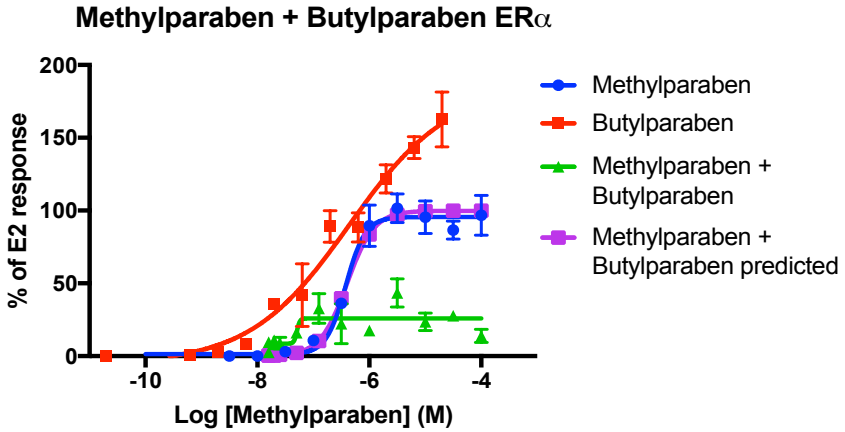

BPA + EE2 ERα
% of E2 response
150
100
50
0
-12
-10
-8
-6
-4
Log [BPA] (M)
BPA
EE2
BPA + EE2
BPA + EE2 predicted

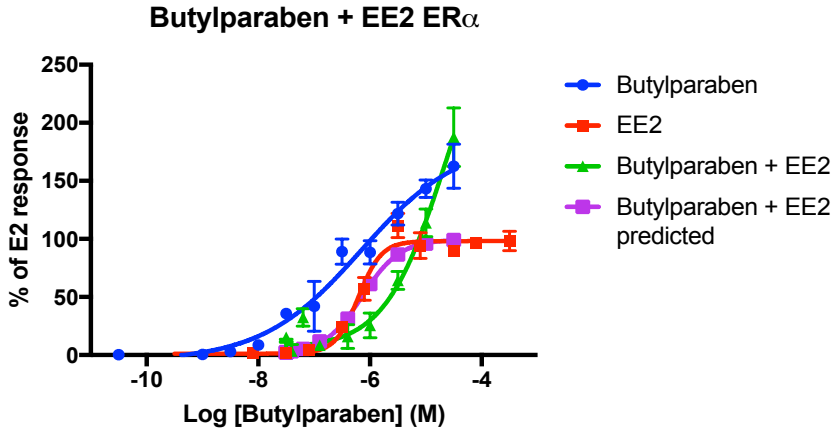

BPA + Methylparaben ERα
% of E2 response
150
100
50
0
-12
-10
-8
-6
-4
Log [BPA] (M)
BPA
Methylparaben
BPA + Methylparaben
BPA + Methylparaben predicted

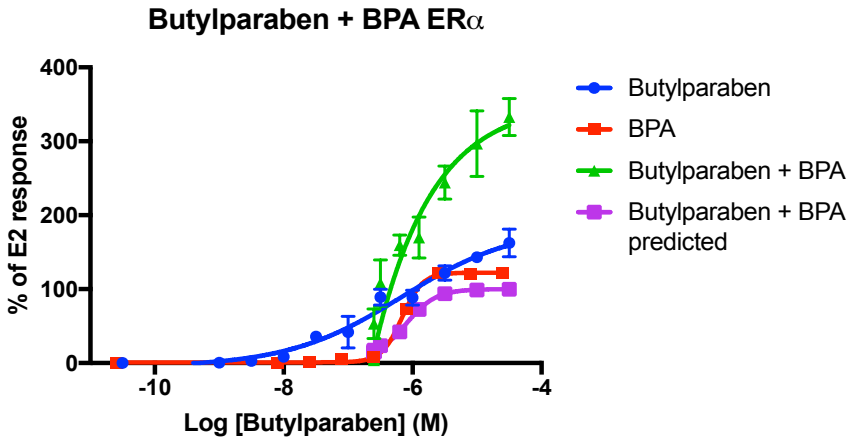

BPA + Butylparaben ERα
% of E2 response
200
150
100
50
0
-12
-10
-8
-6
Log [BPA] (M)
BPA
Butylparaben
BPA + Butylparaben
BPA + Butylparaben predicted

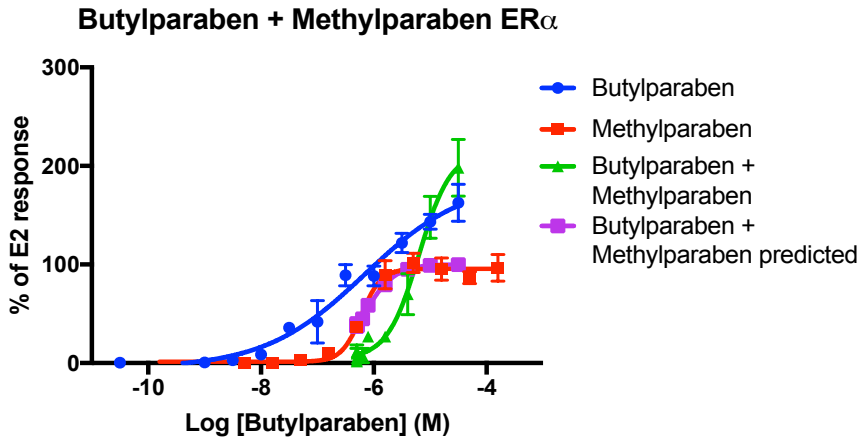

Methylparaben + EE2 ERα
% of E2 response
150
100
50
0
-10
-8
-6
-4
Log [M-para] (M)
Methylparaben
EE2
Methylparaben + EE2
Methylparaben + EE2 predicted


Methylparaben + BPA ERα
% of E2 response
200
150
100
50
0
-50
-10
-8
-6
-4
-2
Log [Methylparaben] (M)
Methylparaben
BPA
Methylparaben + BPA
Methylparaben + BPA predicted

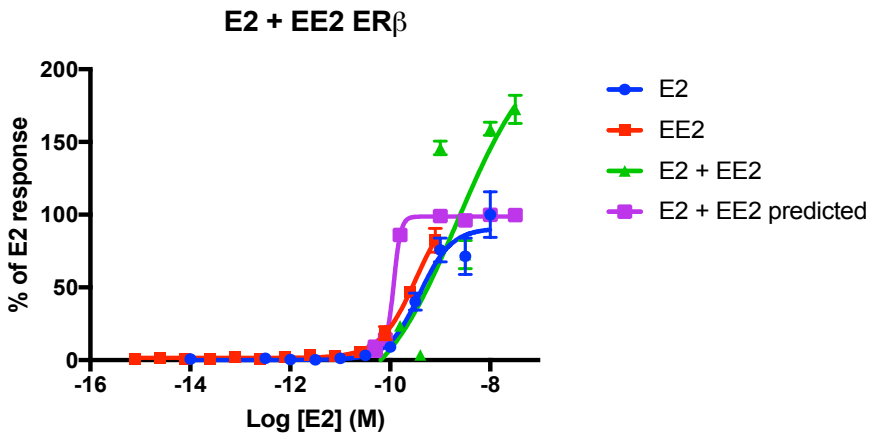

Methylparaben + Butylparaben ERα
% of E2 response
200
150
100
50
0
-10
-8
-6
-4
Log [Methylparaben] (M)
Methylparaben
Butylparaben
Methylparaben + Butylparaben
Methylparaben + Butylparaben predicted

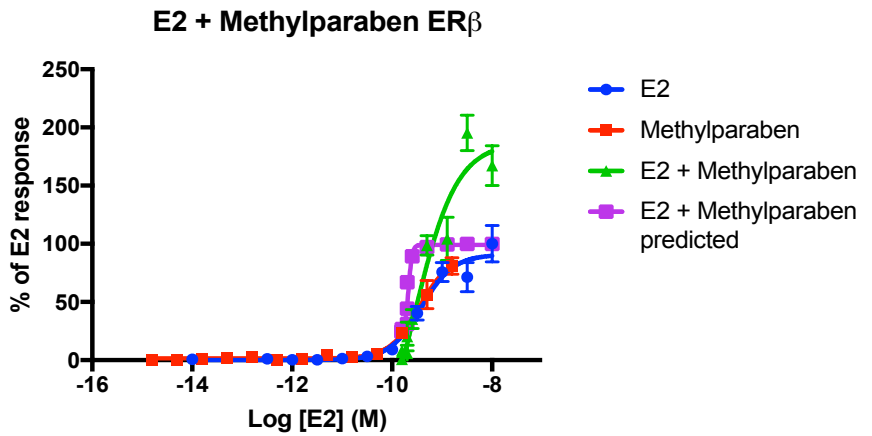

Butylparaben + EE2 ERα
% of E2 response
250
200
150
100
50
0
-10
-8
-6
-4
Log [Butylparaben] (M)
Butylparaben
EE2
Butylparaben + EE2
Butylparaben + EE2 predicted

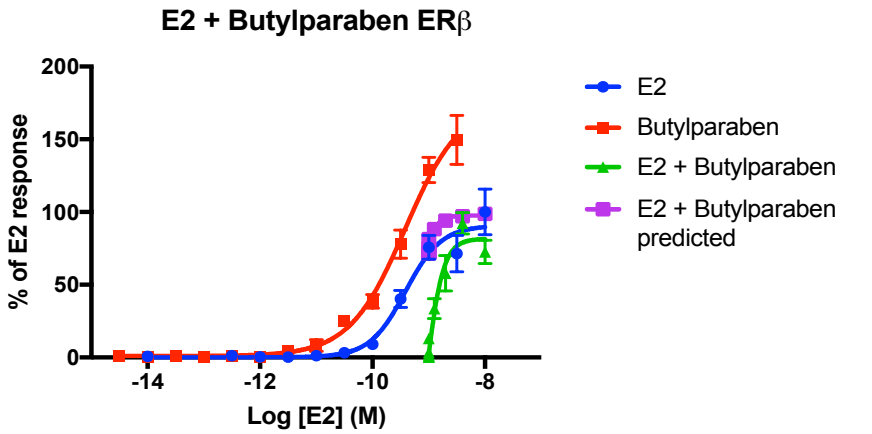

Butylparaben + BPA ERα
% of E2 response
400
300
200
100
0
-10
-8
-6
-4
Log [Butylparaben] (M)
Butylparaben
BPA
Butylparaben + BPA
Butylparaben + BPA predicted

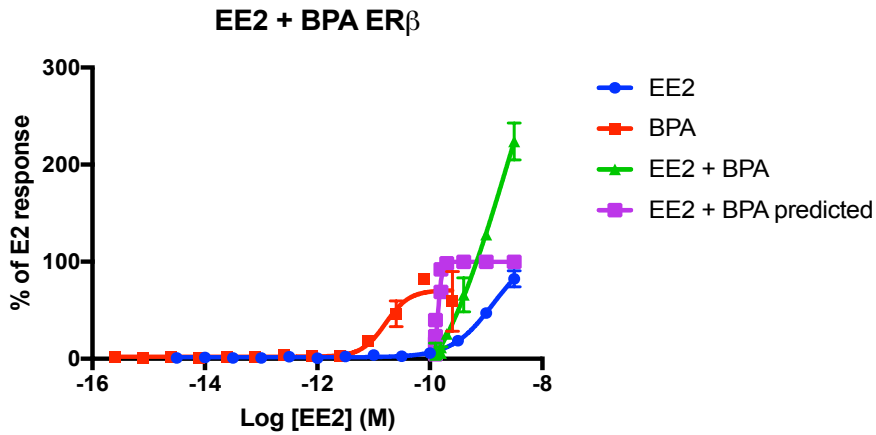

Butylparaben + Methylparaben ERα
% of E2 response
300
200
100
0
-10
-8
-6
-4
Log [Butylparaben] (M)
Butylparaben
Methylparaben
Butylparaben + Methylparaben
Butylparaben + Methylparaben predicted

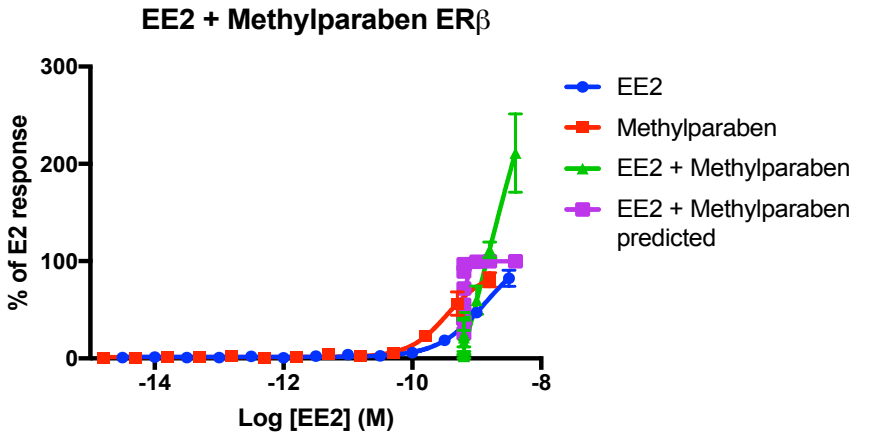
Appendix 2: Results of Individual and 2 Component Combinations of Xenoestrogens in the ERβ CALUX® Assay not Included in Chapter 5. Results are Presented using Non-linear Regression Analysis with Errors Expressed as SEM.

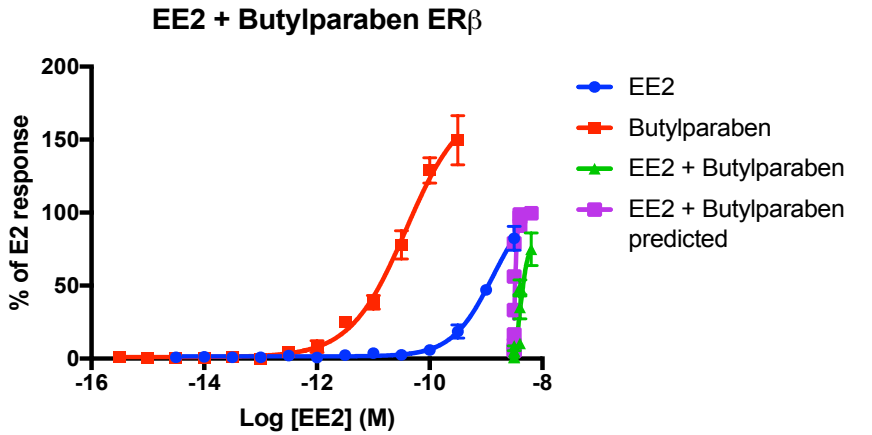

E2 + EE2 ERβ
% of E2 response
200
150
100
50
0
-16
-14
-12
-10
-8
Log [E2] (M)
E2
EE2
E2 + EE2
E2 + EE2 predicted

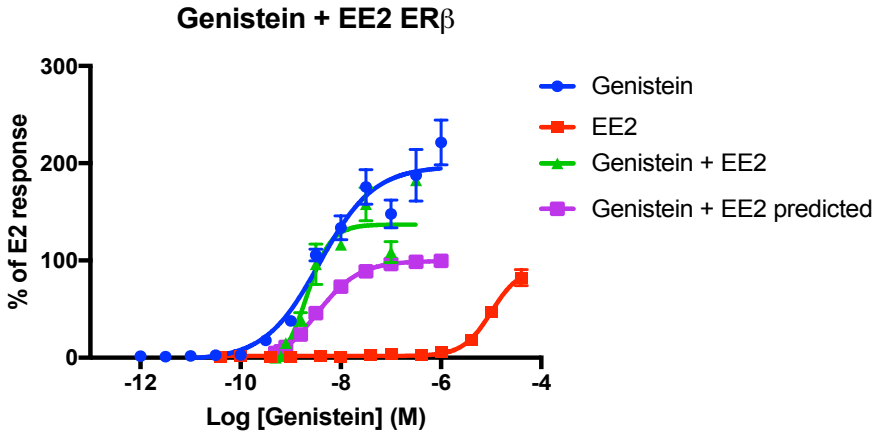

E2 + Methylparaben ERβ
% of E2 response
250
200
150
100
50
0
-16
-14
-12
-10
-8
Log [E2] (M)
E2
Methylparaben
E2 + Methylparaben
E2 + Methylparaben predicted

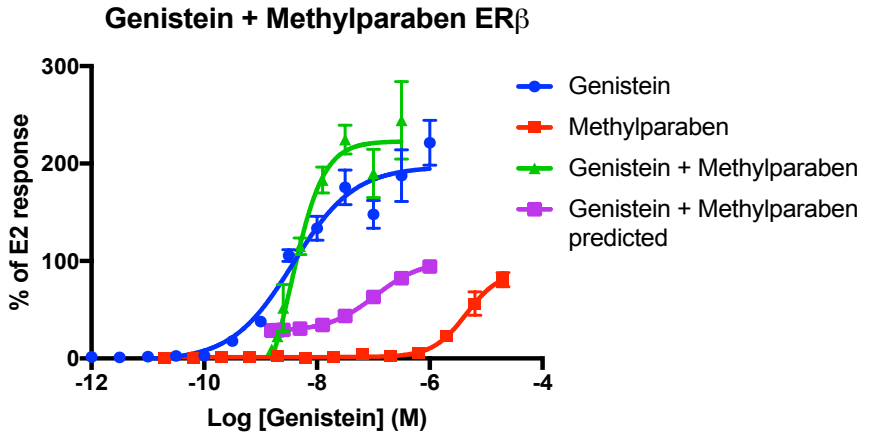

E2 + Butylparaben ERβ
% of E2 response
200
150
100
50
0
-14
-12
-10
-8
Log [E2] (M)
E2
Butylparaben
E2 + Butylparaben
E2 + Butylparaben predicted

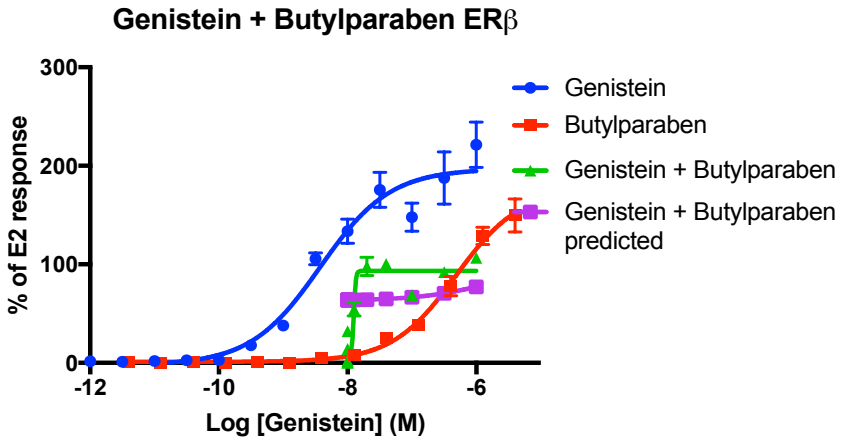

EE2 + BPA ERβ
% of E2 response
300
200
100
0
-16
-14
-12
-10
-8
Log [EE2] (M)
EE2
BPA
EE2 + BPA
EE2 + BPA predicted

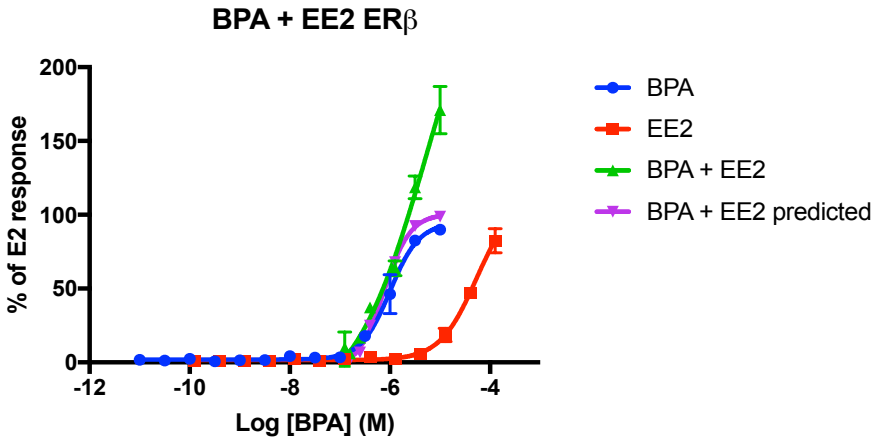

EE2 + Methylparaben ERβ
% of E2 response
300
200
100
0
-14
-12
-10
-8
Log [EE2] (M)
EE2
Methylparaben
EE2 + Methylparaben
EE2 + Methylparaben predicted

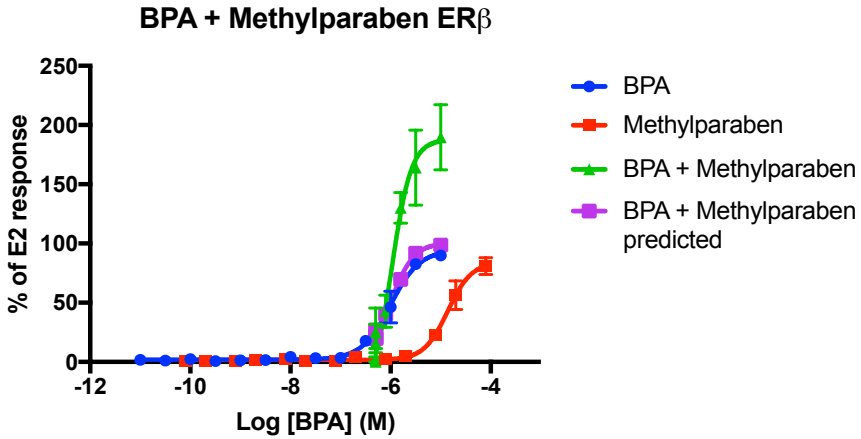

EE2 + Butylparaben ERβ
% of E2 response
200
150
100
50
0
-16
-14
-12
-10
-8
Log [EE2] (M)
EE2
Butylparaben
EE2 + Butylparaben
EE2 + Butylparaben predicted

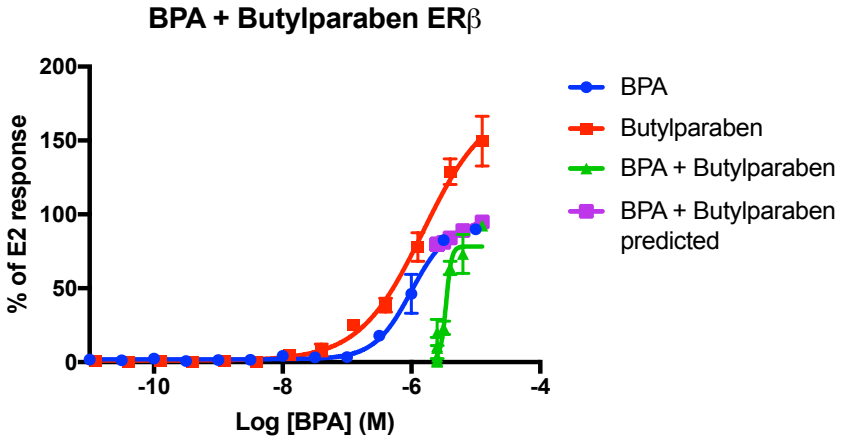

Genistein + EE2 ERβ
% of E2 response
300
200
100
0
-12
-10
-8
-6
-4
Log [Genistein] (M)
Genistein
EE2
Genistein + EE2
Genistein + EE2 predicted

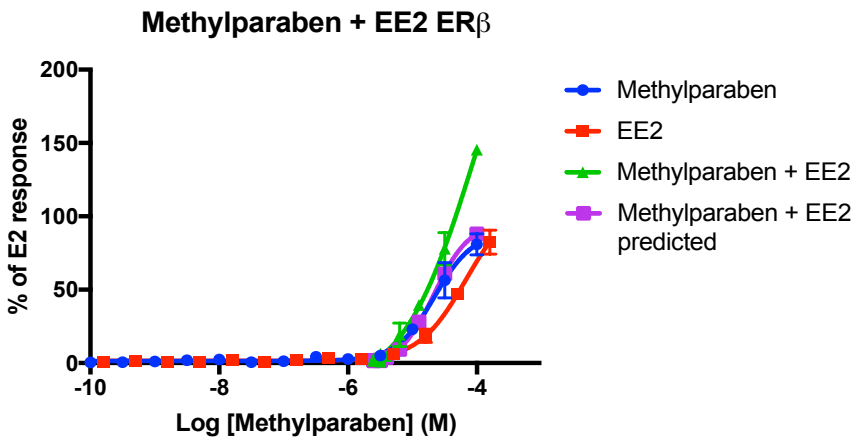

Genistein + Methylparaben ERβ
% of E2 response
300
200
100
0
-12
-10
-8
-6
-4
Log [Genistein] (M)
Genistein
Methylparaben
Genistein + Methylparaben
Genistein + Methylparaben predicted

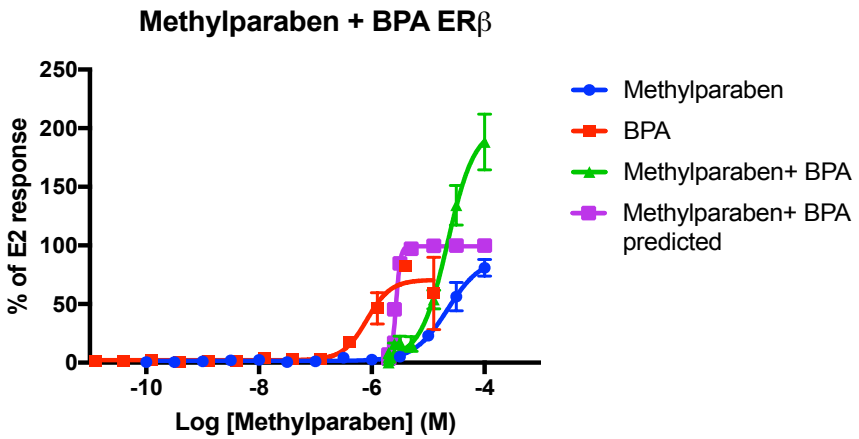

Genistein + Butylparaben ERβ
% of E2 response
300
200
100
0
-12
-10
-8
-6
Log [Genistein] (M)
Genistein
Butylparaben
Genistein + Butylparaben
Genistein + Butylparaben predicted

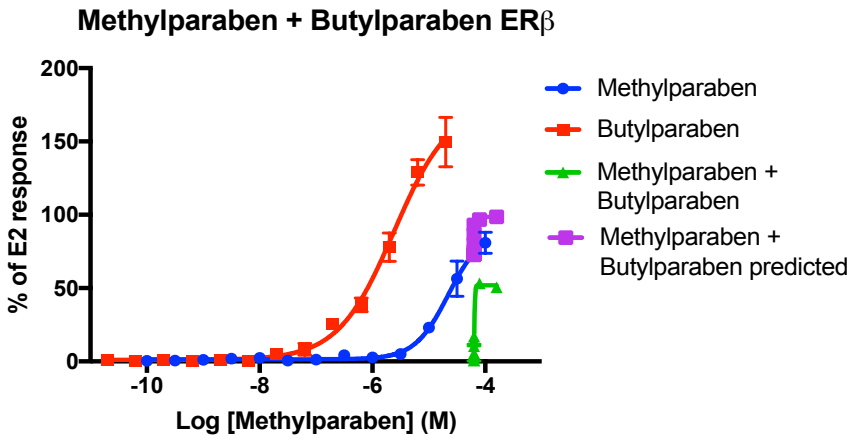

BPA + EE2 ERβ
% of E2 response
200
150
100
50
0
-12
-10
-8
-6
-4
Log [BPA] (M)
BPA
EE2
BPA + EE2
BPA + EE2 predicted

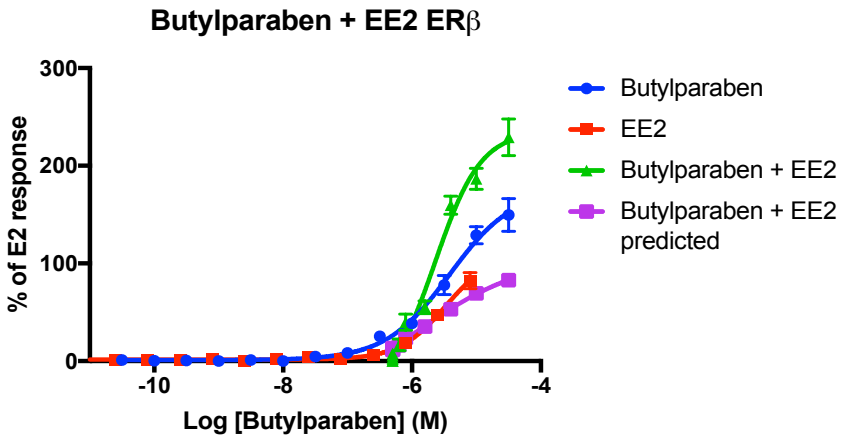

BPA + Methylparaben ERβ
% of E2 response
250
200
150
100
50
0
-12
-10
-8
-6
-4
Log [BPA] (M)
BPA
Methylparaben
BPA + Methylparaben
BPA + Methylparaben predicted

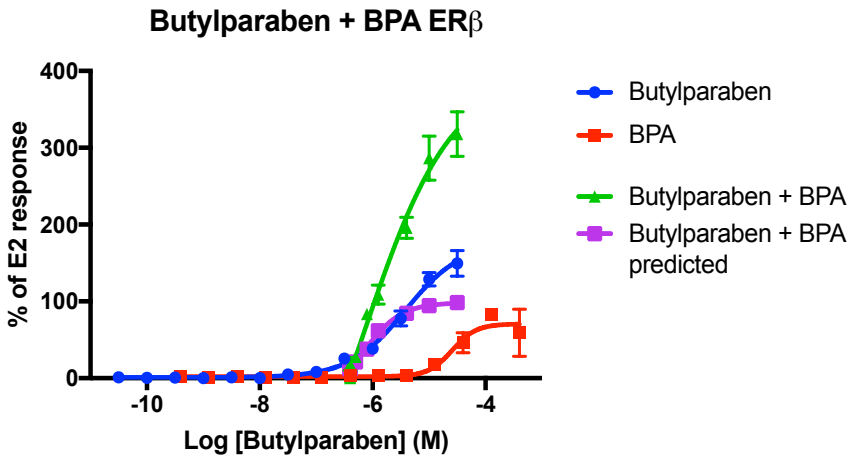

BPA + Butylparaben ERβ
% of E2 response
200
150
100
50
0
-10
-8
-6
-4
Log [BPA] (M)
BPA
Butylparaben
BPA + Butylparaben
BPA + Butylparaben predicted

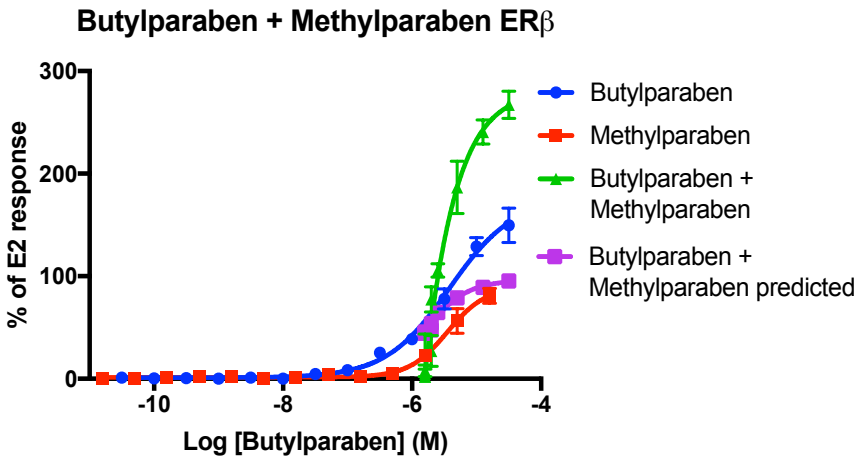

Methylparaben + EE2 ERβ
% of E2 response
200
150
100
50
0
-10
-8
-6
-4
Log [Methylparaben] (M)
Methylparaben
EE2
Methylparaben + EE2
Methylparaben + EE2 predicted


Methylparaben + BPA ERβ
% of E2 response
250
200
150
100
50
0
-10
-8
-6
-4
Log [Methylparaben] (M)
Methylparaben
BPA
Methylparaben+ BPA
Methylparaben+ BPA predicted

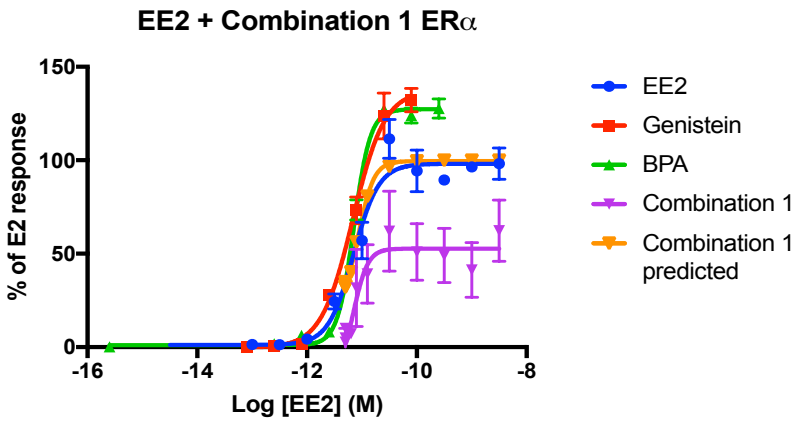

Methylparaben + Butylparaben ERβ
% of E2 response
200
150
100
50
0
-10
-8
-6
-4
Log [Methylparaben] (M)
Methylparaben
Butylparaben
Methylparaben + Butylparaben
Methylparaben + Butylparaben predicted

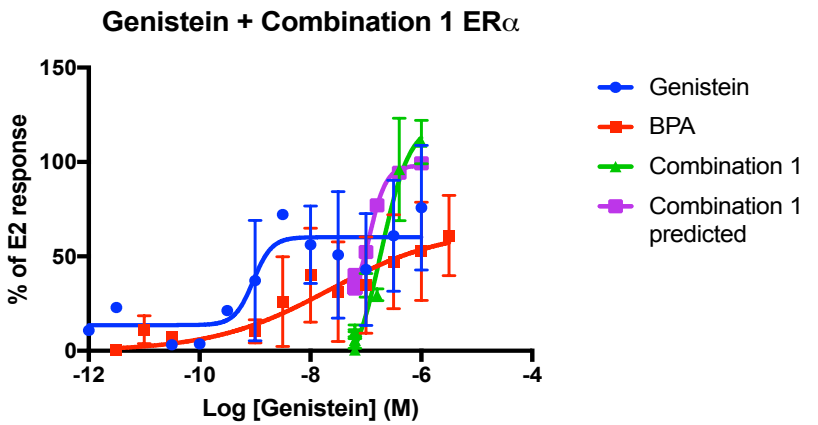

Butylparaben + EE2 ERβ
% of E2 response
300
200
100
0
-10
-8
-6
-4
Log [Butylparaben] (M)
Butylparaben
EE2
Butylparaben + EE2
Butylparaben + EE2 predicted

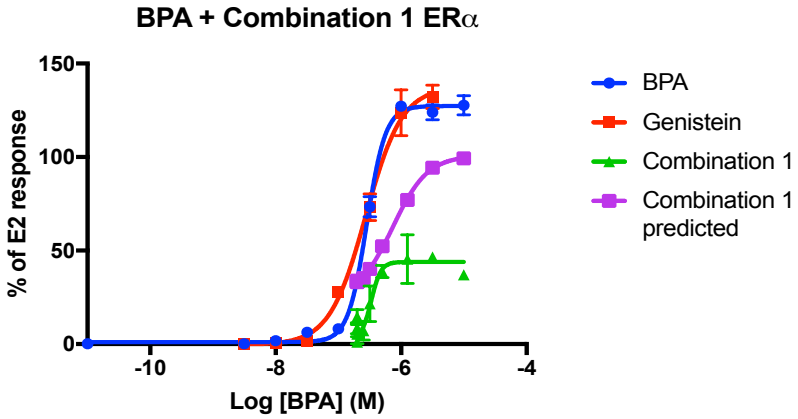

Butylparaben + BPA ERβ
% of E2 response
400
300
200
100
0
-10
-8
-6
-4
Log [Butylparaben] (M)
Butylparaben
BPA
Butylparaben + BPA
Butylparaben + BPA predicted

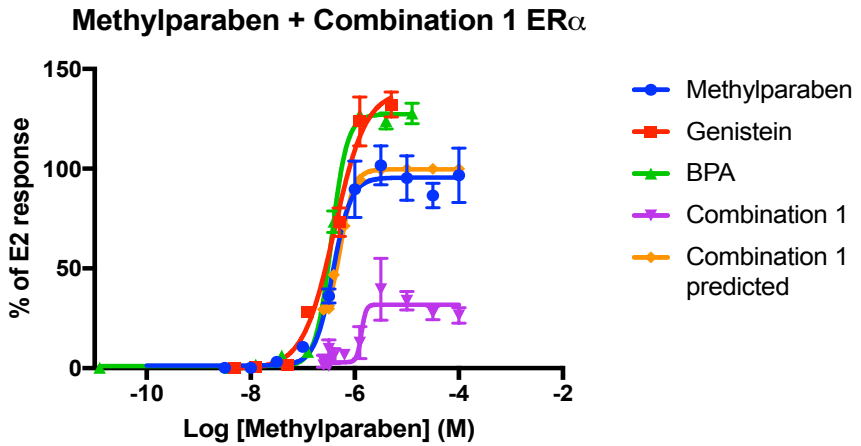

Butylparaben + Methylparaben ERβ
% of E2 response
300
200
100
0
-10
-8
-6
-4
Log [Butylparaben] (M)
Butylparaben
Methylparaben
Butylparaben + Methylparaben
Butylparaben + Methylparaben predicted

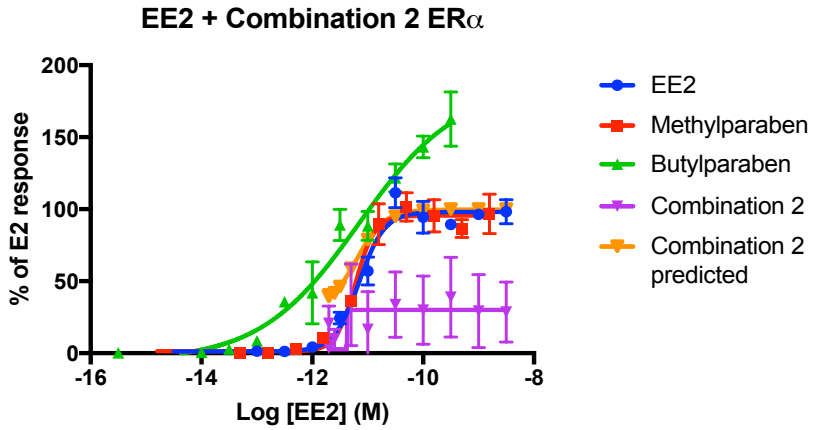
Appendix 3: Results of 3 Component Combinations of Xenoestrogens in the ERα and ERβ CALUX® Assay not Included in Chapter 5. Results are Presented using Non-linear Regression Analysis with Errors Expressed as SEM.

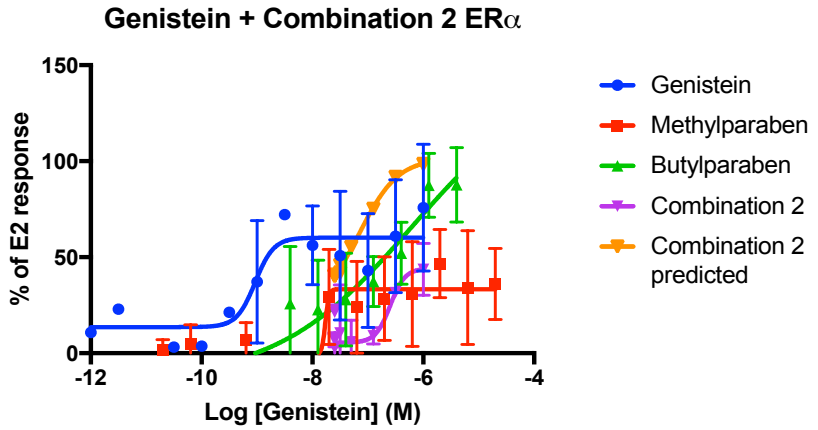

EE2 + Combination 1 ERα
% of E2 response
150
100
50
0
-16
-14
-12
-10
-8
Log [EE2] (M)
EE2
Genistein
BPA
Combination 1
Combination 1 predicted

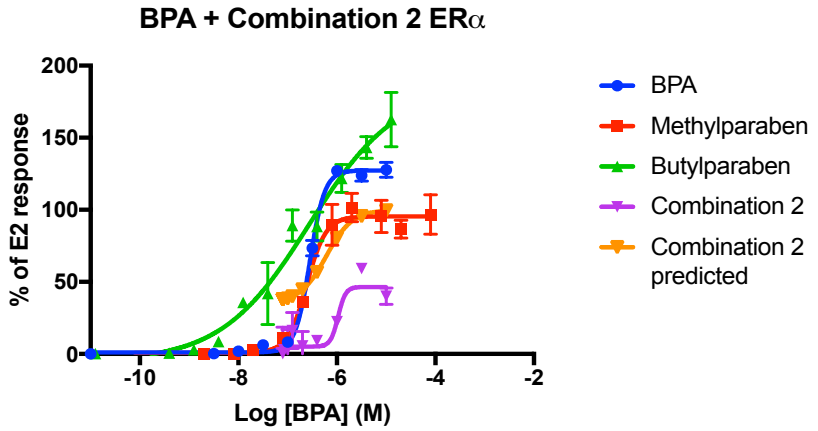

Genistein + Combination 1 ERα
% of E2 response
150
100
50
0
-12
-10
-8
-6
-4
Log [Genistein] (M)
Genistein
BPA
Combination 1
Combination 1 predicted

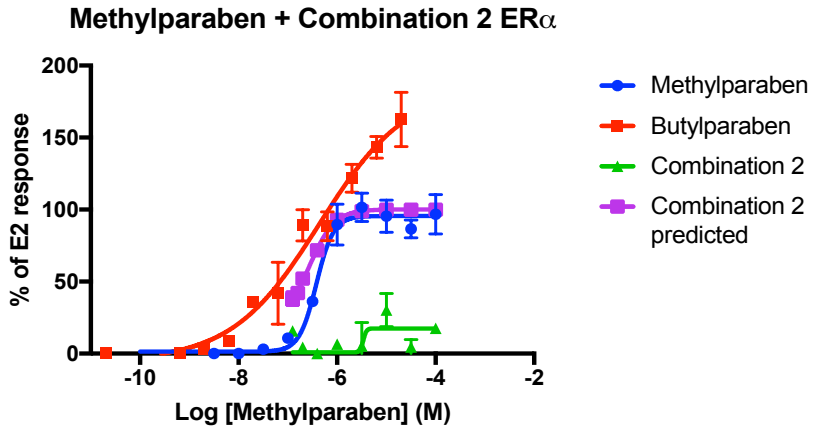

BPA + Combination 1 ERα
% of E2 response
150
100
50
0
-10
-8
-6
-4
Log [BPA] (M)
BPA
Genistein
Combination 1
Combination 1 predicted

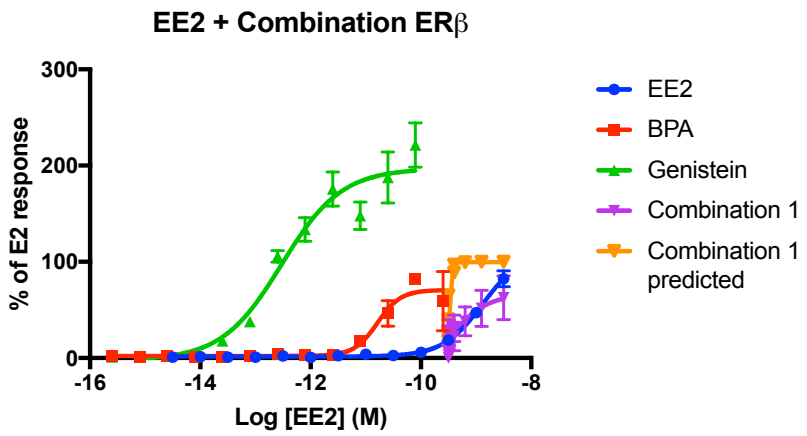

Methylparaben + Combination 1 ERα
% of E2 response
150
100
50
0
-10
-8
-6
-4
-2
Log [Methylparaben] (M)
Methylparaben
Genistein
BPA
Combination 1
Combination 1 predicted

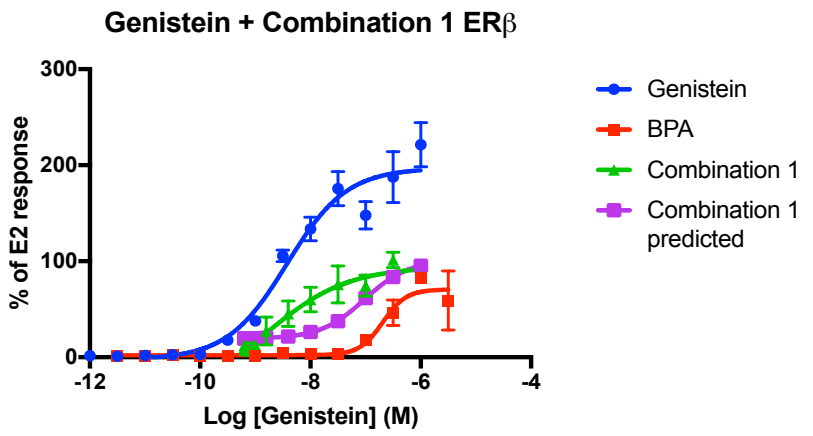

EE2 + Combination 2 ERα
% of E2 response
200
150
100
50
0
-16
-14
-12
-10
-8
Log [EE2] (M)
EE2
Methylparaben
Butylparaben
Combination 2
Combination 2 predicted

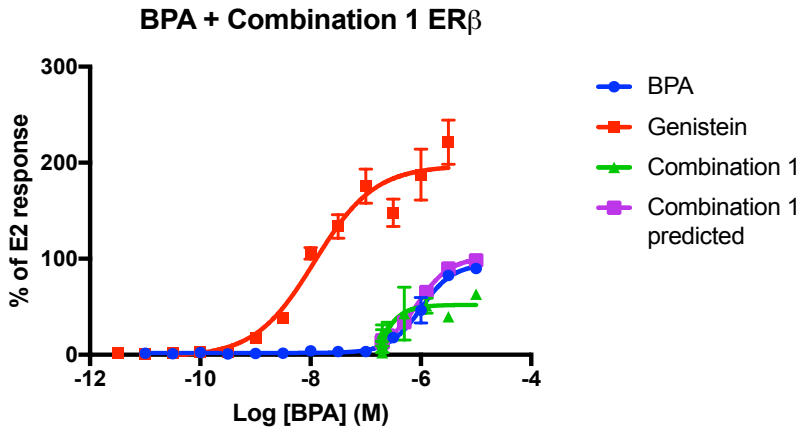

Genistein + Combination 2 ERα
% of E2 response
150
100
50
0
-12
-10
-8
-6
-4
Log [Genistein] (M)
Genistein
Methylparaben
Butylparaben
Combination 2
Combination 2 predicted

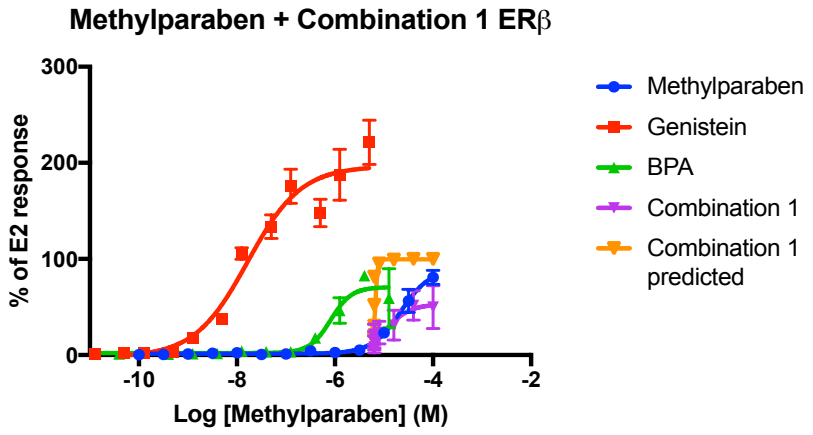

BPA + Combination 2 ERα
% of E2 response
200
150
100
50
0
-10
-8
-6
-4
-2
Log [BPA] (M)
BPA
Methylparaben
Butylparaben
Combination 2
Combination 2 predicted

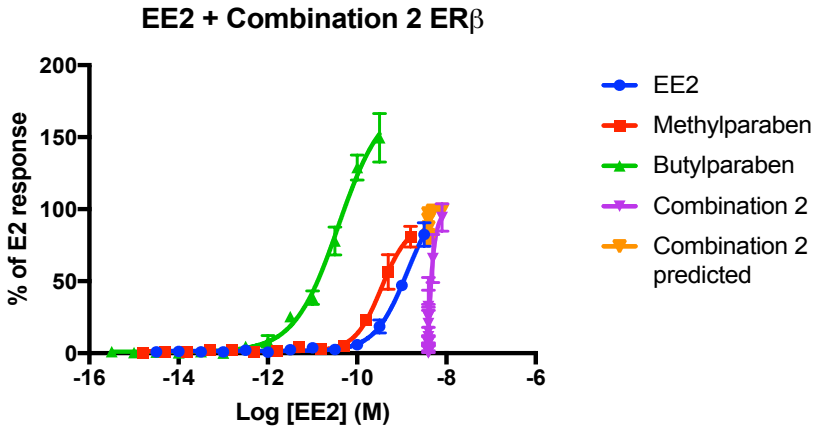

Methylparaben + Combination 2 ERα
% of E2 response
200
150
100
50
0
-10
-8
-6
-4
-2
Log [Methylparaben] (M)
Methylparaben
Butylparaben
Combination 2
Combination 2 predicted

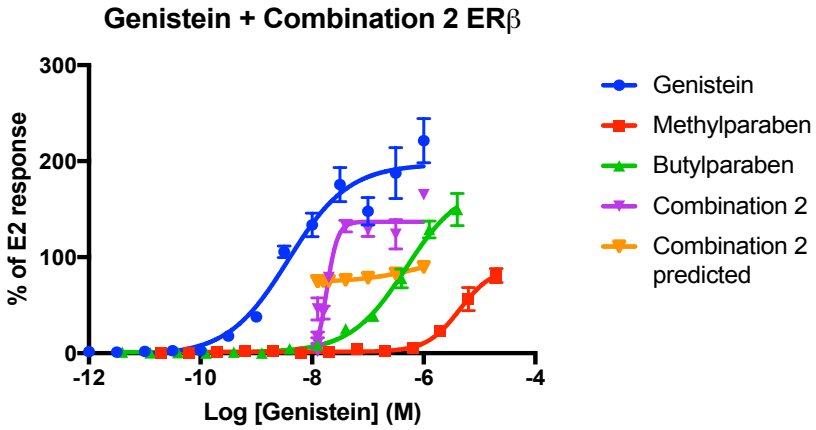

EE2 + Combination ERβ
% of E2 response
300
200
100
0
-16
-14
-12
-10
-8
Log [EE2] (M)
EE2
BPA
Genistein
Combination 1
Combination 1 predicted

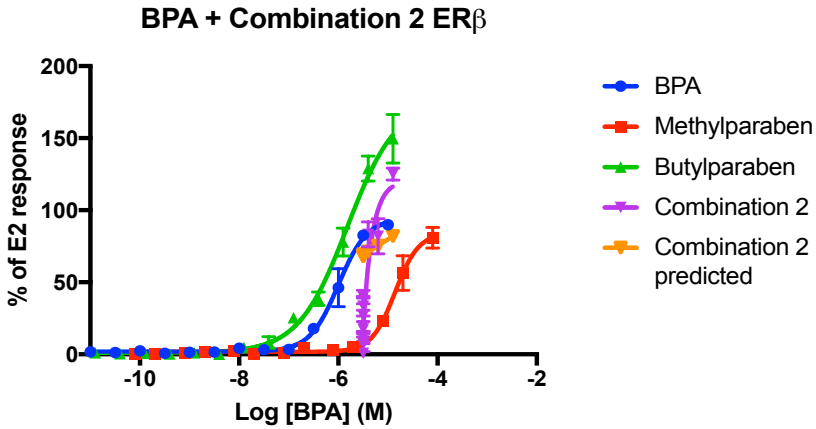

Genistein + Combination 1 ERβ
% of E2 response
300
200
100
0
-12
-10
-8
-6
-4
Log [Genistein] (M)
Genistein
BPA
Combination 1
Combination 1 predicted

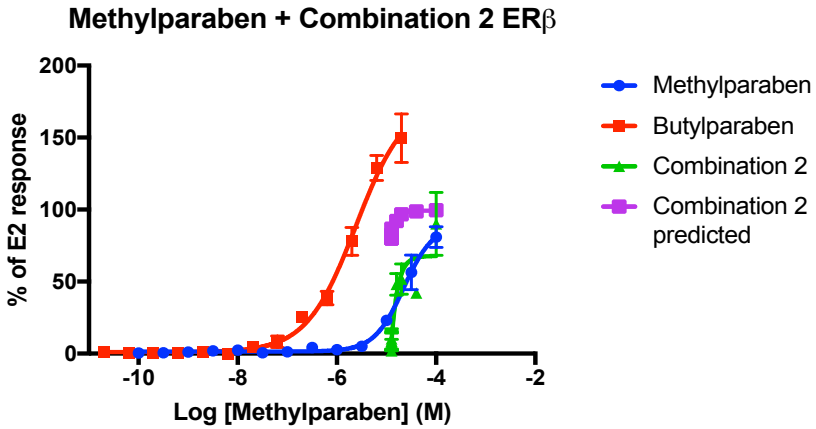

BPA + Combination 1 ERβ
% of E2 response
300
200
100
0
-12
-10
-8
-6
-4
Log [BPA] (M)
BPA
Genistein
Combination 1
Combination 1 predicted


Methylparaben + Combination 1 ERβ
% of E2 response
300
200
100
0
-10
-8
-6
-4
-2
Log [Methylparaben] (M)
Methylparaben
Genistein
BPA
Combination 1
Combination 1 predicted


EE2 + Combination 2 ERβ
% of E2 response
200
150
100
50
0
-16
-14
-12
-10
-8
-6
Log [EE2] (M)
EE2
Methylparaben
Butylparaben
Combination 2
Combination 2 predicted


Genistein + Combination 2 ERβ
% of E2 response
300
200
100
0
-12
-10
-8
-6
-4
Log [Genistein] (M)
Genistein
Methylparaben
Butylparaben
Combination 2
Combination 2 predicted


BPA + Combination 2 ERβ
% of E2 response
200
150
100
50
0
-10
-8
-6
-4
-2
Log [BPA] (M)
BPA
Methylparaben
Butylparaben
Combination 2
Combination 2 predicted


Methylparaben + Combination 2 ERβ
% of E2 response
200
150
100
50
0
-10
-8
-6
-4
-2
Log [Methylparaben] (M)
Methylparaben
Butylparaben
Combination 2
Combination 2 predicted

Appendix 4: List of References for Experimental Binding Energy Measurements used in Chapter 6.

Reference
(Lambrinidis, *et al.*, 2006)
(Lemini, *et al.*, 2003)
(Okubo, *et al.*, 2001)
(Muthyala, *et al.*, 2004)
(Rich, *et al.*, 2002)
(Kuiper, *et al.*, 1997)
(Kuiper, *et al.*, 1998)
(Waller, *et al.*, 1996)
(Mueller, *et al.*, 2004)
(Mueller, *et al.*, 2003)
(Blair, *et al.*, 2000)
(Morohoshi, *et al.*, 2005)
(Zhu, *et al.*, 2006)
(Wolohan, *et al.*, 2004)
(Satoh, *et al.*, 2000)

Appendix 5: List of References for Xenoestrogen Concentration used to Calculate the Daily Xenoestrogen Exposures in Chapter 7.

Reference
(Scott, 2016)
(Fukutake*, et al.*, 1996)
(Guo*, et al.*, 2014)
(Guo*, et al.*, 2014)
(Malekinejad*, et al.*, 2015)
(Kuhnle*, et al.*, 2009)
(Handa*, et al.*, 2009)
(Le*, et al.*, 2008)
(Kubwabo*, et al.*, 2009)
(Nerin*, et al.*, 2003)
(Prabhakaran*, et al.*, 2005)
(Aslam*, et al.*, 2013)
(GolKhoo*, et al.*, 2008)
(Carmichael*, et al.*, 2011)
(Caldwell*, et al.*, 2010)
(Stephany, 2001)

Appendix 6: Information Sheets, Consent Forms, Food and Lifestyle Questionnaire and Daughter's Food and Lifestyle Questionnaire for Women Participating in the Questionnaire and Blood Analysis Studies

Department of Chemistry
Telephone: +64 3 369 3100
Email: samantha.dudley@pg.canterbury.ac.nz

UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

The Role of Xenoestrogen Combinations as Breast Cancer Risk Factors
Information Sheet for Questionnaire Participants

You are invited to participate in a research project studying daily exposure to compounds called environmental estrogens (xenoestrogens). Your participation is entirely voluntary (your choice). You do not have to take part in this study.

Motivation for the Study

There are many compounds in our food and the environment that mimic the natural female hormone estrogen. These mimicking compounds are sometimes known as xenoestrogens and people are exposed to them in their day-to-day lives. It is thought that humans could be exposed to many different combinations of these compounds, where the total exposure may be much higher than anticipated by regulatory authorities. If this is the case, then these compounds may be important in understanding diseases such as breast cancer. Therefore, in this preliminary study we are trying to determine the different combinations of xenoestrogens you may be exposed to in your day-to-day life and whether this is linked to the levels found in your blood, particularly if they could be a potential risk factor for breast cancer development.

Your Participation

If you consent, your participation will be asked in the following:

1. Filling out the questionnaire* about your food and lifestyle habits. The questionnaire will take no longer than 15 minutes to complete.
2. Filling out the questionnaire about your daughters' food and lifestyle habits on her behalf. We ask that you only fill out this questionnaire* if you have a daughter and if she has NOT had her first period. If you have more than one daughter, please only fill out the questionnaire for your oldest daughter that has NOT had her first period. This questionnaire will take no longer than 15 minutes to complete.
3. Please **enclose the signed consent form** when you return the questionnaire/s.

We would appreciate it if you would complete the consent form attached to this information sheet if you are willing to participate in the study. You may keep this information sheet for future reference.

*We have included an online option for the questionnaire. Please type the following links into your search engine and complete the questionnaire on there. Your food and

lifestyle habits questionnaire: http://bit.ly/2n2iSmo and your daughter's food and lifestyle habits questionnaire: http://bit.ly/2nxGKuj.

If you at any time wish to withdraw yourself or your daughter from the study you can contact the researcher Samantha Dudley, Department of Chemistry, University of Canterbury (email: samantha.dudley@pg.canterbury.ac.nz; phone: +64 3 364 6872). Once you have requested to withdraw, all the relevant information pertaining to your, and if applicable your daughter's, involvement will be destroyed within 24 hours. If you decide to to withdraw from the study after your information has been collected, I will remove all information relating to you from my files. However, once your information has been combined with data from other study participants, the information cannot be removed because it is not identifiable.

The Study
This study is being conducted by the University of Canterbury, Department of Chemistry with the co-operation of the University of Canterbury Health Centre and ESR.

The project is being carried out as a requirement of the degree of Doctor of Philosophy in Biochemistry by Samantha Dudley under the supervision of Prof. Ian Shaw and Prof. Ann Richardson who can be contacted at ian.shaw@canterbury.ac.nz & ann.richardson@canterbury.ac.nz. They will be pleased to discuss any concerns you may have about participation in the project.

The results of this project may be published in scientific literature, but you may be assured of complete confidentiality of information gathered in the study. No individual will be identified in any report or publication arising from this research. Only the researcher and named supervisors will have access to the data. Completed questionnaires will be stored securely in a cabinet with a lock and all electronic data will be stored in a password protected computer. The data will be destroyed after 10 years following the completion of my PhD. A thesis is a public document and will be available through the University of Canterbury Library. Alternatively, you may receive a copy of the project results by contacting the researcher at the conclusion of the project.

This project has been reviewed and approved by the University of Canterbury Human Ethics Committee. Participants should address any complaints to The Chair, Human Ethics Committee, University of Canterbury, Private Bag 4800, Christchurch (human-ethics@canterbury.ac.nz).

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UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

The Role of Xenoestrogen Combinations as Breast Cancer Risk Factors
Information Sheet for Questionnaire and Blood Analysis Participants

You are invited to participate in a research project studying daily exposure to compounds called environmental estrogens (xenoestrogens). Your participation is entirely voluntary (your choice). You do not have to take part in this study.

Motivation for the Study

There are many compounds in our food and the environment that mimic the natural female hormone estrogen. These mimicking compounds are sometimes known as xenoestrogens and people are exposed to them in their day-to-day lives. It is thought that humans could be exposed to many different combinations of these compounds, where the total exposure may be much higher than anticipated by regulatory authorities. If this is the case, then these compounds may be important in understanding diseases such as breast cancer. Therefore, in this preliminary study we are trying to determine the different combinations of xenoestrogens you may be exposed to in your day-to-day life and whether this is linked to the levels found in your blood, particularly if they could be a potential risk factor for breast cancer development.

Your Participation

If you consent, your participation will be asked in the following:

4. Filling out the questionnaire about your food and lifestyle habits. The questionnaire will take no longer than 15 minutes to complete.
5. To donate a 20 mL sample. This will be used for the purposes of identifying the levels of xenoestrogens in your blood. In the performance of the venipuncture, there are risks of fainting or feeling light-headed and multiple punctures to locate the veins. A standard blood test is a well-established procedure and these risks are extremely rare.
6. Filling out the questionnaire about your daughters' food and lifestyle habits on her behalf. We ask that you only fill out this questionnaire if you have a daughter and if she has NOT had her first period. If you have more than one daughter, please only fill out the questionnaire for your oldest daughter that has NOT had her first period. This questionnaire will take no longer than 15 minutes to complete.

We would appreciate it if you would complete the consent form attached to this information sheet if you are willing to participate in the study. You may keep this information sheet for future reference.
If you would like any left-over blood to either be returned to you after the study or disposed of with a karakia, please tick the appropriate box on the consent form.

If you at any time wish to withdraw yourself or your daughter from the study you can contact the researcher Samantha Dudley, Department of Chemistry, University of Canterbury (email: samantha.dudley@pg.canterbury.ac.nz; phone: +64 3 364 6872). Once you have requested to withdraw, all the relevant information pertaining to your, and if applicable your daughter's, involvement will be destroyed within 24 hours. If you decide to to withdraw from the study after your information has been collected, I will remove all information relating to you from my files. However, once your information has been combined with data from other study participants, the information cannot be removed because it is not identifiable.

The Study
This study is being conducted by the University of Canterbury, Department of Chemistry with the co-operation of the University of Canterbury Health Center and ESR.

The project is being carried out as a requirement of the degree of Doctor of Philosophy in Biochemistry by Samantha Dudley under the supervision of Prof. Ian Shaw and Prof. Ann Richardson who can be contacted at ian.shaw@canterbury.ac.nz & ann.richardson@canterbury.ac.nz. They will be pleased to discuss any concerns you may have about participation in the project.

The results of this project may be published in scientific literature, but you may be assured of complete confidentiality of information gathered in the study. No individual will be identified in any report or publication arising from this research. Only the researcher and named supervisors will have access to the data. Completed questionnaires will be stored securely in a cabinet with a lock and all electronic data will be stored in a password protected computer. The data will be destroyed after 10 years following the completion of my PhD. A thesis is a public document and will be available through the University of Canterbury Library. Alternatively, you may receive a copy of the project results by contacting the researcher at the conclusion of the project.

This project has been reviewed and approved by the University of Canterbury Human Ethics Committee. Participants should address any complaints to The Chair, Human Ethics Committee, University of Canterbury, Private Bag 4800, Christchurch (human-ethics@canterbury.ac.nz).

Samantha Dudley
Research leader
Email: samantha.dudley@pg.canterbury.ac.nz
Phone: +64 3 364 6872

Department of Chemistry
Telephone: +64 3 369 3100
Email: samantha.dudley@pg.canterbury.ac.nz

UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

The Role of Xenoestrogen Combinations as Breast Cancer Risk Factors
Consent Form for Questionnaire

I…….. consent to the information provided by me about myself, and if applicable my daughter, to be used in this study. I understand what is required of me and that my participation is voluntary.

I understand that if I choose to complete the questionnaire online, pressing the submit button is equivalent to signing this consent form. I am consenting to the data provided by me to be used in the study for the purposes outlined in the information sheet.

I understand that the data obtained from the studies of my questionnaire will be confidential and all reports written utilizing the data will not include my name or any other information that would identify my daughter or myself.

All personal information relating to the information provided will be kept confidential and not disclosed to anyone outside the researcher and nominated supervisors. All data will be stored for 10-years in a locked facility.

I understand that at any time if I wish to withdraw myself or my daughter from the study I can contact the researcher Samantha Dudley, Department of Chemistry, University of Canterbury (email: samantha.dudley@pg.canterbury.ac.nz; phone: +64 3 369 3100). Once you have requested to withdraw, all the relevant information pertaining to your, and if applicable your daughter's, involvement will be destroyed within 24 hours. If you decide to withdraw from the study after your information has been collected, your information will be removed from the files. However, once your information has been combined with data from other study participants, the information cannot be removed because it is not identifiable.

If you would like a copy of the final report of the findings, please tick this box ☐

I understand that I can contact the researcher Samantha Dudley, Department of Chemistry, University of Canterbury (email: samantha.dudley@pg.canterbury.ac.nz; phone: +64 3 369 3100) or supervisors Prof. Ian Shaw and Prof. Ann Richardson (email: ian.shaw@canterbury.ac.nz & ann.richardson@canterbury.ac.nz; phone: +64 3 369 4302 & +64 3 369 3520) for further information. If I have any complaints, I can contact the Chair of the University of Canterbury Human Ethics Committee, Private Bag 4800, Christchurch (human-ethics@canterbury.ac.nz).

Signed…….

Date…….

Department of Chemistry
Telephone: +64 3 364 6872
Email: samantha.dudley@pg.canterbury.ac.nz

UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

The Role of Xenoestrogen Combinations as Breast Cancer Risk Factors
Consent Form for Questionnaire and Blood Analysis

I……consent to a 20 mL blood sample being taken from a vein in my arm and that the blood can be used to measure estrogens and estrogen mimicking compounds. I also consent to the information provided by me either about myself and if applicable my daughter to be used in this study.

The risks associated with the taking a blood sample are very low, but I accept these as part of my involvement in the study.

I understand that the data obtained from the studies of my questionnaire and blood will be confidential and all reports written utilizing the data will not include my name or any other information that would identify myself.

All personal information relating linked to the information provided and sample I give will be kept confidential and not disclosed to anyone outside the researcher and nominated supervisors. All data will be stored for 10-years in a locked facility.

I understand that at any time if I wish to withdraw myself or my daughter from the study I can contact the researcher Samantha Dudley, Department of Chemistry, University of Canterbury (email: samantha.dudley@pg.canterbury.ac.nz; phone: +64 3 364 6872). Once you have requested to withdraw, all the relevant information pertaining to your, and if applicable your daughter's, involvement will be destroyed within 24 hours. If you decide to withdraw from the study after your information has been collected, your information will be removed from the files. However, once your information has been combined with data from other study participants, the information cannot be removed because it is not identifiable.

If you would like a copy of the final report of the findings please tick this box ☐
If you would like any left-over blood disposed with a karakia please tick this box ☐
If you would like any left-over blood returned after the study please tick this box ☐

I understand that I can contact the researcher Samantha Dudley, Department of Chemistry, University of Canterbury (email: samantha.dudley@pg.canterbury.ac.nz; phone: +64 3 364 6872) or supervisors Prof. Ian Shaw and Prof. Ann Richardson (email: ian.shaw@canterbury.ac.nz & ann.richardson@canterbury.ac.nz; phone: +64 3 364 3105 & +64 3 369 3520) for further information. If I have any complaints, I can contact the Chair of the University of Canterbury Human Ethics Committee, Private Bag 4800, Christchurch (human-ethics@canterbury.ac.nz).

Signed……..
Date……..

Questionnaire about your Food and Lifestyle Habits

Xenoestrogens can be found in food (e.g. bread), plastics (e.g. wine bottle caps, plastic containers), personal care products (e.g. make-up) and some medicines. The combinations of these compounds that humans are exposed to on a daily basis could have some long term health effects. The information you provide in this questionnaire will be used to understand the different combinations of xenoestrogens and to calculate the total estrogenic load people are being exposed on a daily basis.

Name: _____

Date of Birth: _____

Food and Drink

Consumption

1. How many slices of bread do you usually eat per day?

   ☐ less than 1 per day
   ☐ 1 slice per day
   ☐ 2 slices per day
   ☐ 3 slices per day
   ☐ 4 slices per day
   ☐ 5 slices per day
   ☐ 6 or more slices per day (please specify) _____
   ☐ I don't know or I'd prefer not to answer

2. What sort of bread do you most frequently eat? If you eat speciality bread (e.g. ciabatta) please include it in the other section.

   ☐ I don't eat bread
   ☐ white bread
   ☐ wholemeal bread
   ☐ multigrain bread
   ☐ soy and linseed-containing bread (e.g. Vogel's soy and linseed)
   ☐ other (please specify) _____
   ☐ I don't know or I'd prefer not to answer

3. How often do you eat soy based products (e.g. tofu, soy yogurt, etc.)?

   ☐ I don't eat soy based products
   ☐ less than once a week
   ☐ 1-2 times a week
   ☐ 3-4 times a week
   ☐ 5-6 times a week
   ☐ once a day
   ☐ 2 or more times a day
   ☐ I don't know or I'd prefer not to answer

4. How often do you eat fresh eggs (e.g. eggs in a carton)?

- ☐ I don't eat eggs
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day
- ☐ I don't know or I'd prefer not to answer

5. How often do you eat alternative egg products (e.g. powdered eggs, packaged egg whites, etc.)?

- ☐ I don't eat alternative egg products
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day
- ☐ I don't know or I'd prefer not to answer

6. How often do you eat any of the following vegetables?

	Never	Less than once a **month**	Once a **month**	2 or more times a **month**	1-2 times a **week**	3-4 times a **week**	5-6 times a **week**	Once a **day**	2-3 times a **day**	More than 3 times a **day**	I don't know or I prefer not to answer
Spinach											
Carrots											
Yams											
Parsnip											

7. How often do you eat the following fruits?

	Never	Less than once a **month**	Once a **month**	2 or more times a **month**	1-2 times a **week**	3-4 times a **week**	5-6 times a **week**	Once a **day**	2-3 times a **day**	More than 3 times a **day**	I don't know or I prefer not to answer
Apple											
Pear											
Orange											
Kiwifruit											

8. What type of milk do you most frequently usually drink?

- ☐ I don't drink milk
- ☐ cows milk
- ☐ goats milk
- ☐ almond milk
- ☐ rice milk
- ☐ soy milk
- ☐ other (please specify) _____
- ☐ I don't know or I'd prefer not to answer

9. How much milk do you usually drink on average per day? Note 1 glass = 250 mL

- ☐ none
- ☐ less than 1 glass
- ☐ 1-2 glasses
- ☐ 3-4 glasses
- ☐ 5 or more glasses (please specify) _____
- ☐ I don't know or I'd prefer not to answer

10. How often do you eat the following dairy products? Please DO NOT include non-dairy based products e.g. coconut ice cream.

	Never	Less than once a **month**	Once a **month**	2 or more times a **month**	1-2 times a **week**	3-4 times a **week**	5-6 times a **week**	Once a **day**	2-3 times a **day**	More than 3 times a **day**	I don't know or I prefer not to answer
Butter/margarine											
Cheese											
Yoghurt											
Ice cream											
Cream											

11. How often do you eat the following meat?

	Never	Less than once a **month**	Once a **month**	2 or more times a **month**	1-2 times a **week**	3-4 times a **week**	5-6 times a **week**	Once a **day**	2-3 times a **day**	More than 3 times a **day**	I don't know or I prefer not to answer
Beef											
Chicken											
Lamb											
venison											
Fish/seafood											

12. How many glasses of water from the tap do you drink on average per day (1 glass = 250mL)?

- ☐ I don't drink water from the tap
- ☐ less than 1 glass
- ☐ 1 glass
- ☐ 2 glasses
- ☐ 3 glasses
- ☐ 4 glasses
- ☐ 5 glasses
- ☐ 6 glasses
- ☐ 7 glasses
- ☐ 8 glasses
- ☐ more than 8 glasses (please specify) _____
- ☐ I don't know or I'd prefer not to answer

13. How many canned drinks do you on average drink per day (e.g. Sprite, Red Bull, etc.)?

- ☐ I don't drink canned drinks
- ☐ less than 1 can
- ☐ 1-2 cans
- ☐ 3-4 cans
- ☐ 5-6 cans
- ☐ 7 or more cans (please specify) _____
- ☐ I don't know or I'd prefer not to answer

14. Do you drink alcohol out of a screw top bottle (e.g. a bottle of wine)?

- ☐ yes
- ☐ no
- ☐ I don't know or I'd prefer not to answer

15. How many glasses of alcohol would you drink on average per day? Note 1 glass = 250 mL

- ☐ none
- ☐ less than 1 glass
- ☐ 1-2 glasses
- ☐ 3-4 glasses
- ☐ 5 or more glasses (please specify) _____
- ☐ I don't know or I'd prefer not to answer

Packaging

16. How often do you microwave food in a plastic container?

- ☐ Never
- ☐ less than once a week
- ☐ 1-2 times a week

- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day
- ☐ I don't know or I'd prefer not to answer

17. How much drink on average do you usually consume out of a plastic bottle per day (e.g. sprite, orange juice, water etc.)? Note: 1 cup = 250 mL.

- ☐ none
- ☐ less than 1 cup
- ☐ 1-2 cups
- ☐ 3-4 cups
- ☐ 5-6 cups
- ☐ 7 or more cups
- ☐ I don't know or I'd prefer not to answer

18. Do you ever leave plastic bottles filled with drink in the sun (e.g. a water bottle in your car)?

- ☐ yes - please specify how often you would do this _____
- ☐ no
- ☐ I don't know or I'd prefer not to answer

19. How often do you drink out of a can?

- ☐ never
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day
- ☐ I don't know or I'd prefer not to answer

20. How often do you wrap your food in cling wrap?

- ☐ never
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day (please specify) _____
- ☐ I don't know or I'd prefer not to answer

21. How often do you eat food that has been packaged in a plastic wrapper (e.g. a muesli bar, meat etc.)?

- ☐ never
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day (please specify) _____
- ☐ I don't know or I'd prefer not to answer

22. How often do you eat food out of a tin can (e.g. baked beans, tomatoes etc.)?

- ☐ never
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day (please specify) _____
- ☐ I don't know or I'd prefer not to answer

23. How often do you put your plastic containers through a wash cycle in a dish washer? Plastic containers include lunch boxes, storage containers etc. We do NOT want you to include plastic trays or food packaging etc.

- ☐ never
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ I don't know or I'd prefer not to answer

Travel

24. Have arrived back from overseas in the last week?

- ☐ yes
- ☐ no
- ☐ I don't know or I'd prefer not to answer

If your answer is yes, could you tell us the country you most recently visited in the space provided below.

Please specify here:

_____

_____

_____

Tablets and Pills

25. Do you currently take any form of contraceptive pill?

☐ yes
☐ no
☐ I don't know or I'd prefer not to answer

If your answer is yes, can you please provide the name of the pill you are taking in the space provided below.

Please specify here:

_____
_____
_____
_____

26. Are you currently on any other form of hormone contraceptive (e.g. progesterone implant, Depo Provera injection or IUD)?

☐ yes
☐ no
☐ I don't know or I'd prefer not to answer

If your answer is yes, please can you provide the name of the hormone contraceptive you are on in the space provided below.

Please specify here:

_____
_____
_____

27. Are you currently on any form of hormone replacement therapy?

☐ yes
☐ no
☐ I don't know or I'd prefer not to answer

If your answer is yes, please can you proved the name of hormone replacement therapy you are on in the space provided below.

Please specify here:

_____
_____
_____

28. Are you currently on any form of fertility treatment?

☐ yes
☐ no
☐ I don't know or I'd prefer not to answer

If your answer is yes, please can you provide the name of fertility treatment you are on in the space provided below.

Please specify here:

_____
_____
_____

29. Do you take any dietary supplements?

☐ yes
☐ no
☐ I don't know or I'd prefer not to answer

If your answer is yes, could you please provide the name and brand of the supplement in the space provided below. Dietary supplements can include products such as multivitamins purchased from the supermarket.

Please specify here:

_____
_____
_____

Personal Care Products

30. How often do you use any of the following personal care products? Please note that using once a day does not include re-application (e.g. if you put lipstick on in the morning and re-apply it twice throughout the day, then your daily use is 3 times in total).

	Never	Less than once a **month**	Once a **month**	2 or more times a **month**	1-2 times a **week**	3-4 times a **week**	5-6 times a **week**	Once a **day**	2-3 times a **day**	More than 3 times a **day**	I don't know or I prefer not to answer
Foundation											
Mascara											
Lipstick											
Sunblock											
Moisturiser											
Face cleanser											
Nail polish											
Deodorant											
Shampoo											
Conditioner											
Make-up remover											
Spray tan											

Medication

31. Have you taken any medication in the past week?

☐ yes
☐ no
☐ I don't know or I'd prefer not to answer

If your answer is yes, could you please specify the name of the medication and whether you are on it long term (longer than 2 weeks) or short term (e.g. less than two weeks) in the space provided below. Medications can include anything from regular medication (e.g. warfarin) to Panadol or Nurofen.

Please specify here:

_____
_____
_____

32. Have you had a tooth filling in the last week?

☐ yes
☐ no
☐ I don't know or I'd prefer not to answer

Breast Cancer

33. Have you had or do you have breast cancer?

☐ yes
☐ no
☐ I don't know or I'd prefer not to answer

If your answer is yes, please can you tell us whether it is estrogen receptor positive or estrogen receptor negative in the space provided below.

Please specify here:

_____
_____
_____

34. Before participating in this survey had you thought about estrogen mimicking compounds being a risk factor for breast cancer?

☐ I had not heard of estrogen mimicking compounds before this survey
☐ yes
☐ no
☐ I don't know or I'd prefer not to answer

35. If you were previously aware of some of these estrogen mimicking compounds, did you take any measures to eliminate them from your daily routine (e.g. purchase paraben free make-up or BPA free containers)?

☐ yes
☐ no
☐ I had not heard of estrogen mimicking compounds before this survey
☐ I don't know or I'd prefer not to answer

If your answer is yes, could you please explain to us what measures you have taken to eliminate estrogen mimics from your daily routine in the space provided below.

Please specify here:

_____
_____
_____

Preferred contact details
Please note you do not have to provide any details you are not comfortable with.

Phone: _____
Address:

_____
Email:

_____

Weight: _____ kgs

Occupation:

_____
_____
_____

Questionnaire about your Daughters Food and Lifestyle Habits

Xenoestrogens can be found in food (e.g. bread), plastics (e.g. wine bottle caps, plastic containers), personal care products (e.g. make-up) and some medicines. The combinations of these compounds that humans are exposed to on a daily basis could have some long term health effects. The information you provide in this questionnaire will be used to understand the different combinations of xenoestrogens and to calculate the total estrogenic load your daughters are being exposed on a daily basis. It is important to consider pre-puberty exposure in females because they are more susceptible to the influences of estrogen mimicking compounds due to their own low natural estrogen levels. It is also thought that exposure to the compounds pre-puberty may increase their risk of developing breast cancer later in life. We ask that you fill out this survey if your daughter has NOT had her first period. If you have more than one daughter, please fill out the questionnaire for your oldest daughter that has NOT had her first period.

Date of Birth: _____

Food and Drink

Consumption

1. How many slices of bread does your daughter usually eat per day?

   ☐ less than 1 per day
   ☐ 1 slice per day
   ☐ 2 slices per day
   ☐ 3 slices per day
   ☐ 4 slices per day
   ☐ 5 slices per day
   ☐ 6 or more slices per day (please specify) _____
   ☐ I don't know or I'd prefer not to answer

2. What sort of bread does your daughter most frequently eat? If she eats speciality bread (e.g. ciabatta) please include it in the OTHER section.

   ☐ She doesn't eat bread
   ☐ white bread
   ☐ wholemeal bread
   ☐ multigrain bread
   ☐ soy and linseed-containing bread (e.g. Vogel's soy and linseed)
   ☐ other (please specify) _____
   ☐ I don't know or I'd prefer not to answer

3. How often does your daughter eat soy based products (e.g. tofu, soy yogurt, etc.)?

- ☐ She doesn't eat soy based products
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day
- ☐ I don't know or I'd prefer not to answer

4. How often does your daughter eat fresh eggs (e.g. eggs in a carton)?

- ☐ She doesn't eat eggs
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day
- ☐ I don't know or I'd prefer not to answer

5. How often does your daughter eat alternative egg products (e.g. powdered eggs, packaged egg whites, etc.)?

- ☐ She doesn't eat alternative egg products
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day
- ☐ I don't know or I'd prefer not to answer

6. How often does your daughter eat any of the following vegetables?

	Never	Less than once a **month**	Once a **month**	1-2 times a **week**	3-4 times a **week**	Once a **day**	1-2 times a **day**	3 or more times a **day**	I don't know or prefer not to answer
Spinach									
Carrot									
Yams									
Parsnip									

7. How often does your daughter eat the following fruits?

	Never	Less than once a **month**	Once a **month**	1-2 times a **week**	3-4 times a **week**	Once a **day**	1-2 times a **day**	3 or more times a **day**	I don't know or prefer not to answer
Apple									
Pear									
Orange									
Kiwifruit									

8. What type of milk does your daughter most frequently drink?

☐ She doesn't drink milk
☐ cows milk
☐ goats milk
☐ almond milk
☐ rice milk
☐ soy milk
☐ other (please specify) _____
☐ I don't know or I'd prefer not to answer

9. How much milk does your daughter usually drink on average per day? Note 1 glass = 250 mL

☐ none
☐ less than 1 glass
☐ 1-2 glasses
☐ 3-4 glasses
☐ 5 or more glasses (please specify) _____
☐ I don't know or I'd prefer not to answer

10. How often does your daughter eat the following dairy products? Please DO NOT include non-dairy based products e.g. coconut ice cream.

	Never	Less than once a **month**	Once a **month**	1-2 times a **week**	3-4 times a **week**	Once a **day**	1-2 times a **day**	3 or more times a **day**	I don't know or prefer not to answer
Butter/margarine									
Cheese									
Yogurt									
Ice cream									
Cream									

11. How often does your daughter eat the following meat?

	Never	Less than once a **month**	Once a **month**	1-2 times a **week**	3-4 times a **week**	Once a **day**	1-2 times a **day**	3 or more times a **day**	I don't know or prefer not to answer
Beef									
Chicken									
Lamb									
Venison									
Fish/seafood									

12. How many glasses of water from the tap does your daughter drink on average per day (1 glass = 250mL)?

- ☐ She doesn't drink water from the tap
- ☐ less than 1 glass
- ☐ 1 glass
- ☐ 2 glasses
- ☐ 3 glasses
- ☐ 4 glasses
- ☐ 5 glasses
- ☐ 6 glasses
- ☐ 7 glasses
- ☐ 8 glasses
- ☐ more than 8 glasses (please specify) _____
- ☐ I don't know or I'd prefer not to answer

13. How many canned drinks does your daughter on average drink per day (e.g. Sprite, Red Bull, etc.)?

- ☐ She doesn't drink canned drinks
- ☐ less than 1 can
- ☐ 1-2 cans
- ☐ 3-4 cans
- ☐ 5-6 cans
- ☐ 7 or more cans (please specify) _____
- ☐ I don't know or I'd prefer not to answer

Packaging

14. How often does your daughter eat food microwaved in a plastic container?

- ☐ Never
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day
- ☐ I don't know or I'd prefer not to answer

15. How much drink on average does your daughter usually consume out of a plastic bottle per day (e.g. sprite, orange juice, water etc.)? Note: 1 cup = 250 mL.

- ☐ none
- ☐ less than 1 cup
- ☐ 1-2 cups
- ☐ 3-4 cups
- ☐ 5-6 cups
- ☐ 7 or more cups
- ☐ I don't know or I'd prefer not to answer

16. How often does your daughter eat food wrapped in cling wrap?

- ☐ never
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day (please specify) _____
- ☐ I don't know or I'd prefer not to answer

17. How often does your daughter eat food that has been packaged in a plastic wrapper (e.g. a muesli bar, meat etc.)?

- ☐ never
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day (please specify) _____
- ☐ I don't know or I'd prefer not to answer

18. How often does your daughter eat food out of a tin can (e.g. baked beans, tomatoes etc.)?

- ☐ never
- ☐ less than once a week
- ☐ 1-2 times a week
- ☐ 3-4 times a week
- ☐ 5-6 times a week
- ☐ once a day
- ☐ 2 or more times a day (please specify) _____
- ☐ I don't know or I'd prefer not to answer

19. How often does your daughter eat the following canned foods?

	Never	Less than once a **month**	Once a **month**	1-2 times a **week**	3-4 times a **week**	Once a **day**	1-2 times a **day**	3 or more times a **day**	I don't know or prefer not to answer
Salmon									
Tuna									
Other tinned meats									
Coconut cream									
Olives									

Travel

20. Has your daughter arrived back from overseas in the last week?

- ☐ yes
- ☐ no
- ☐ I don't know or I'd prefer not to answer

If your answer is yes, could you tell us the country she most recently visited in the space provided below.

Please specify here:

_____

_____

_____

Tablets and Pills

21. Does your daughter take any dietary supplements?

☐ yes
☐ no
☐ I don't know or I'd prefer not to answer

If your answer is yes, could you please provide the name and brand of the supplement in the space provided below. Dietary supplements can include products such as multivitamins purchased from the supermarket.

Please specify here:

_____

_____

_____

Personal Care Products

22. How often does your daughter use any of the following personal care products? Please note that using once a day does not include re-application

*Clearly, some of these products are not necessarily applicable to younger girls.

	Never	Less than once a **month**	Once a **month**	2 or more times a **month**	1-2 times a **week**	3-4 times a **week**	5-6 times a **week**	Once a **day**	2-3 times a **day**	More than 3 times a **day**	I don't know or I prefer not to answer
Foundation											
Mascara											
Lipstick											
Sunblock											
Moisturiser											
Face cleanser											
Nail polish											
Deodorant											
Shampoo											
Conditioner											
Make-up remover											
Spray tan											

Medication

23. Has your daughter taken any medication in the past week?

☐ yes
☐ no
☐ I don't know or I'd prefer not to answer

If your answer is yes, could you please specify the name of the medication and whether your daughter is on it long term (longer than 2 weeks) or short term (e.g. less than two weeks) in the space provided below. Medications can include anything from regular medication (e.g. warfarin) to Panadol or Nurofen.

Please specify here:

_____
_____
_____

24. Has your daughter had a tooth filling in the last week?

☐ yes
☐ no
☐ I don't know or I'd prefer not to answer

25. Before participating in this survey had you thought about estrogen mimicking compounds being a risk factor for breast cancer for your daughter?

☐ I had not heard of estrogen mimicking compounds before this survey
☐ yes
☐ no
☐ I don't know or I'd prefer not to answer

26. If you were previously aware of some of these estrogen mimicking compounds, did you take any measures to eliminate them from your daughter's daily routine (e.g. purchase BPA free containers)?

☐ yes
☐ no
☐ I had not heard of estrogen mimicking compounds before this survey
☐ I don't know or I'd prefer not to answer

If your answer is yes, could you please explain to us what measures you have taken to eliminate estrogen mimics from your daily routine in the space provided below.

Please specify here:

_____
_____
_____
_____

Appendix 7: Human Ethics Committee Approval for the Studies

HUMAN ETHICS COMMITTEE

Secretary, Rebecca Robinson Telephone: +64 03 364 2987, Extn 45588 Email: human-ethics@canterbury.ac.nz

Ref: HEC 2016/45 25 July 2016

Samantha Dudley
Chemistry
UNIVERSITY OF CANTERBURY

Dear Samantha

The Human Ethics Committee advises that your research proposal "The Role of Xenoestrogen Combinations as Breast Cancer Risk Factors" has been considered and approved.

Please note that this approval is subject to the incorporation of the amendments you have provided in your email of 20th July 2016.

Best wishes for your project.

Yours sincerely

R. Robinson

pp.

Jane Maidment

Chair
University of Canterbury Human Ethics Committee

Appendix 8: MCF-7 Individual Data

Compound/s	Mean (cells/mL)	SEM
Control	9.5×10^5	2.7×10^4
E2	1.2×10^6	1.8×10^4
EE2	1.4×10^6	1.3×10^4
BPA	1.1×10^6	2.4×10^4
Genistein	1.1×10^6	1.8×10^4
Butylparaben	1.5×10^6	1.8×10^4
Tetrahydrocurcumin	1.2×10^6	1.2×10^4
Kaempferol	1.1×10^6	1.4×10^4
Estriol	1.2×10^6	3.1×10^4
EE2 + E2	1.2×10^6	1.9×10^4
BPA + E2	1.1×10^6	1.0×10^4
Genistein + E2	6.5×10^5	8.7×10^3
Butylparaben + E2	8.8×10^5	2.4×10^4
Tetrahydrocurcumin + E2	6.6×10^5	6.9×10^3
Kaempferol + E2	5.9×10^5	2.5×10^4
Estriol + E2	6.3×10^5	2.2×10^4
Combination	9.5×10^5	1.0×10^4

Appendix 9: Publications Arising from this Thesis

List of Publications

Ye, H., Dudley, S. Z., and Shaw, I. C. (2017). *Escherichia coli* biotransformation of daidzein fermentation products from soy-based foods – relevance to food oestrogenicity-based functionality. *International Journal of Food Science and Technology.* **52**, 1082-1091.

Ye, H., Dudley, S. Z., and Shaw, I. C. (2018). Intimate estrogen receptor-α/ligand relationships signal biological activity. *Toxicology*. **408**, 80-87.